UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Zoología y Antropología Física

TESIS DOCTORAL

Molecular characterization of lizard parasites and their influence on colour ornaments

Caracterización molecular de parásitos que infectan lagartos y su influencia sobre los ornamentos de color

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Rodrigo Manuel Megía Palma

Directores
Santiago Merino Rodríguez
Javier Martínez González

Madrid, 2016

MOLECULAR CHARACTERIZATION OF LIZARD PARASITES AND THEIR INFLUENCE ON COLOUR ORNAMENTS

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Departamento de Ecología Evolutiva
Museo Nacional de Ciencias Naturales
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Departamento de Zoología y Antropología Física
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“The beginning of wisdom is calling things by their right names”.
(Confucius, ca. 500 BC)

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Memoria presentada por el licenciado Rodrigo Manuel Megía Palma para optar al grado de doctor en Ciencias Biológicas por la Universidad Complutense de Madrid. Dirigida por los directores Santiago Merino Rodríguez, profesor científico del Museo Nacional de Ciencias Naturales del Consejo Superior de Investigaciones Científicas y Javier Martínez González, profesor contratado doctor en la Universidad de Alcalá de Henares.

Santiago Merino Rodríguez Javier Martínez González

Fdo. El Doctorando Vº. B. Directores

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Gallotia galloti insulanagae, Roque de Fuera (Tenerife)

Cortesía de **Iván Acevedo**

“Only by understanding the environment and how it works can we make the necessary decisions to protect it.

Only by evaluating all our precious natural and human resources can we hope to build a sustainable world”

(UN Secretary-General Kofi Annan, 30 Mar 2005).

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Ahora parece que está todo hecho, y el camino no ha hecho más que empezar…

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RESUMEN

La presente tesis doctoral ha abordado dos objetivos principales: 1) la caracterización morfológica y molecular de los protozoos eimeriorinos más prevalentes en lagartos (*Schellackia*, *Lankesterella*, *Acroeimeria*, *Choleoeimeria*, *Caryospora* e *Isospora*), así como su análisis filogenético y 2) el efecto que ejercen sobre la expresión de los ornamentos de color en diferentes sistemas parásito-hospedador.

Para llevar a cabo el primer objetivo se tomaron muestras de heces y tejido sanguíneo de lagartos silvestres y también de lagartos presentes en tiendas especializadas. Las muestras sanguíneas fueron obtenidas mediante la punción de la base de la cola con una aguja estéril. La sangre extraída se utilizó para realizar un diagnóstico directo, mediante frotis sanguíneo, y uno molecular a partir de las muestras conservadas en tarjetas FTA-Whatman. Las muestras para el estudio de los parásitos intestinales se obtuvieron a partir de heces recogidas de la cloaca de los hospedadores. Tras la homogeneización de la muestra, una parte se utilizó para realizar el diagnóstico coprológico mientras que otra parte fue empleada para la identificación molecular de los parásitos detectados mediante la amplificación del gen nuclear *18S* ribosomal. Una vez caracterizados molecularmente los coccidios hemáticos o intestinales, se procedió a la realización de un análisis filogenético para esclarecer las relaciones evolutivas existentes entre estos géneros, desconocidos en gran medida, de coccidios. Los resultados más relevantes de este primer bloque fueron:

1) La obtención de la primera caracterización molecular del género *Schellackia*.

2) La identificación y caracterización de 18 haplotipos diferentes de parásitos del género *Schellackia* en 15 de las 17 especies de lagartos de la familia Lacertidae estudiados en la Península Ibérica y el Norte de África.

3) La separación de la familia Lankesterellidae en dos familias monogenéricas y, como consecuencia, la revalidación de la familia Schellackiidae Grassé 1953.

4) La obtención de la primera caracterización molecular de parásitos del género *Isospora* y *Caryospora* en reptiles.

5) La obtención de soporte molecular para dar validez a los géneros *Choleoeimeria* y *Acroeimeria* que infectan los conductos biliares y el intestino, respectivamente.

El segundo objetivo abordado en la tesis ha estado relacionado con el estudio del papel jugado por alguno de los coccidios, previamente caracterizados, en la expresión de los ornamentos visuales de los hospedadores. La intención final fue testar la hipótesis de Hamilton y

Zuk (1982). Esta hipótesis predice que poblaciones bajo una elevada presión selectiva ejercida por enfermedades parasitarias expresarían ornamentos o comportamientos sexuales complejos. Además, los individuos podrían señalizar su estado de infección y su calidad genética en términos de resistencia a las infecciones parasitarias mediante la expresión diferencial de sus ornamentos sexuales secundarios. Para ello, se seleccionaron tres sistemas diferentes de parásito-hospedador. El primer estudio fue llevado a cabo en las Islas Canarias, explorando el efecto de la intensidad de parásitos del género *Karyolysus* (Apicomplexa: Adeleorina) sobre un ornamento sexual dicromático en machos y hembras del lagarto tizón *Gallotia galloti* (Squamata: Lacertidae). El segundo estudio fue llevado a cabo con el lagarto verdinegro *Lacerta schreiberi* (Squamata: Lacertidae) en la provincia de Segovia, relacionando la presencia de parásitos (*Schellackia*, nematodos y ectoparásitos) con la coloración de la garganta. En el tercer sistema parásito/hospedador, se comprobó el efecto de los géneros de coccidios *Acroeimeria* (Apicomplexa: Eimeriorina) y *Schellackia* (Apicomplexa: Eimeriorina) sobre ciertos ornamentos de color presentes en los lagartos de valla *Sceloporus occidentalis bocourtii* (Squamata: Phrynosomatidae) en California. Como se indica en el primer objetivo, en cada estudio se tomaron muestras para poder detectar y cuantificar las infecciones. Por otra parte, se empleó espectrofotometría para cuantificar de una manera objetiva los parámetros de color de cada uno de los ornamentos expresados por los lagartos. Los resultados más relevantes obtenidos en este segundo objetivo fueron:

1) La infección por *Karyolysus* y *Schellackia* fue relacionada positivamente con el croma de ornamentos azules en dos especies de lagartos de la familia Lacertidae, *G. galloti* y *L. schreiberi*.

2) La infección por *Schellackia* se relacionó negativament con el brillo de un ornamento ventral azul en los machos de *S. occidentalis bocourtii*.

3) El brillo del ornamento amarillo de las patas delanteras de las hembras de *S. occidentalis bocourtii* infectadas por *Acroeimeria* fue mayor que en los machos infectados. Sin embargo, no hubo diferencias sexuales cuando los individuos no estaban infectados. Aun así, este parámetro estuvo relacionado negativamente con la condición en ambos sexos.

Conclusiones

1) Los géneros *Schellackia* y Lankesterella tienen orígenes evolutivos independientes y, por tanto, la familia Lankesterellidae no tiene un origen monofilético.

2) El género *Schellackia* es mucho más diverso y específico de lo que se creía con anterioridad. De hecho los diferentes géneros de lagartos de la familia Lacertidae de la Península Ibérica no compartieron haplotipos de este hemococcidio aun cuando las especies son simpátricas.

3) Los representantes del género *Isospora* aislados en reptiles no están relacionados filogenéticamente con los detectados en aves o mamíferos, por lo que podría tratarse como un género independiente.

4) El género *Caryospora* no es monofilético, ya que la caracterización molecular de un aislamiento procedente de lagarto estuvo más estrechamente relacionado con el género *Lankesterella* que con las secuencias de *Caryospora* aisladas en ratones.

5) Los parásitos aislados en lagartos cuyos ooquistes son morfológicamente similares a los del género *Eimeria* forman un clado monofilético propio de reptiles. Además, los análisis filogenéticos validarían el uso de los géneros *Acroeimeria* y *Choleoeimeria* propuestos inicialmente por Paperna y Landsberg (1989) que se basaron en la morfología de los ooquistes.

6) Las relaciones encontradas entre la carga o la presencia de endoparásitos con la expresión de ornamentos de color azul in distintos sistemas parásito-hospedador son compatibles con un mayor depósito de eumelanina en la piel de las lagartos estudiados. Dado que se requiere de altas condiciones oxidativas para favorecer la síntesis de eumelanina, señales azules o azules-ultravioletas pueden estar relacionadas con la capacidad individual de resistir el estrés oxidativo de manera similar a otros vertebrados que también muestran ornamentos basados en melaninas.

7) Los ornamentos de color amarillo se pueden ver afectados tanto por infecciones crónicas (endoparásitos) como por infecciones agudas y estacionales (ectoparásitos).

8) En especies donde ambos sexos están ornamentados de manera similar, la respuesta fenotípica a la infección por parásitos puede ser en sentido opuesto.

9) En poblaciones naturales con alta incidencia de parasitismo, el dimorfismo cromático es "recompensado" en términos de condición corporal y grado de infección. Por ejemplo, las "mujeres barbudas", refiriéndose a hembras con rasgos típicamente masculinos, sufren un hándicap en la naturaleza. De este modo, las hembras de la lagartija americana *Sceloporus occidentalis bocourtii*, y del lagarto canario, *Gallotia galloti palmae* estuvieron en mejor condición física o estuvieron más infrecuentemente parasitadas cuando mostraron rasgos típicamente femeninos. Por otro lado, los machos con rasgos de color más vistosos, típicos de machos dominantes, deben de reflejar una mejor calidad individual en línea con la hipótesis del hándicap propuesta por Zahavi.

SUMMARY

This dissertation achieved two main objectives: 1) the morphologic, molecular and phylogenetic characterization of the most prevalent eimeriorine protozoan found in reptiles (*Schellackia, Lankesterella, Acroeimeria, Choleoeimeria, Caryospora and Isospora*) and, 2) the effect of the parasitosis produced by some of these pathogens over the conspicuousness of coloured ornaments in different lizard host-parasite systems.

For the first objective, we got blood and fecal samples from both free-ranging and captive lizards from pet stores. We obtained the blood samples from the coccygeal vein with a steril needle. This blood was used for a direct diagnostic by both microscopy and molecular screening (we targeted the *18S* rRNA gene). Similarly, the screening of the fecal samples was used for diagnosing intestinal coccidiosis by both microscopy and molecular amplification of *18S* rRNA gene. After the molecular characterization of hematic and intestinal coccidia, we included the sequences from these parasites in Bayesian and Maxima likelyhood phylogenetic trees to understand the evolutionary affinities among these genera of coccidia. The main results for this part were:

1) We obtained the first molecular characterization for genus *Schellackia*.

2) The identification and molecular characterization of 18 different haplotypes of parasites of the genus *Schellackia* from 15 of the 17 lacertid species that we studied from the Iberian Peninsula and North Africa.

3) Based on molecular results, we propose splitting the family Lankesterellidae in two different monogeneric families and, in consequence, the re-erection of family Schellackiidae Grassé 1953.

4) We obtained the first molecular characterization of parasites in the genera *Isospora* and *Caryospora* that infect reptiles.

5) We obtained good phylogenetic support to validate the genera *Choleoeimeria* and *Acroeimeria* (Paperna & Landsberg 1989) that undergo their endogenous development in the billiar ducts and the intestine, respectively.

The second objetive achieved in this thesis was related with the role that some coccidia play in the expression of coloured ornaments of lizard hosts. Our aim was testing Hamilton and Zuk's hypothesis (1982). This hypothesis predicts that populations subjected to high selective pressure by parasitic diseases may evolve complex sexual ornaments and/or displays. In addition, the individuals may signal their status of infection and their genetic quality in relation with their capability to stand infectious diseases through the conspicuity of their secondary sexual

characters. With this purpose, we select three different host-parasite systems. The first study was perfomed in the Canary Islands where we explored the effect of the load of parasites of the genus *Karyolysus* (Apicomplexa: Adeleorina) on a dichromatic sexual ornament (blue and whitish) in the cheek of males and females *Gallotia galloti*. The second study was performed on *Lacerta schreiberi* in a population from Segovia. In this study, we explored the relation of the presence of hemococcidia genus *Schellackia* (Apicomplexa: Eimeriorina), nematoda and tick load with two colour patches (blue and yellow) in the throught of lizard hosts. In the third host-parasite system, we studied the effect of parasites of the genera *Acroeimeria* (Apicomplexa: Eimeriorina) and *Schellackia* on ventral ornaments (blue and yellow) of fence lizards, *Sceloporus occidentalis bocourtii* (Squamata: Phrynosomatidae) from California. As commented in the first objective, we obtained samples in each of the systems of study in order to diagnose and quantify the infections. In the orther hand, we used spectrophotometric technology to quantify objectively the colour of the visual ornaments of the lizards. The main results of these second part were:

1) The infection by *Karyolysus* and *Schellackia* was positively related with the chroma of blue ornaments in two lizards of the family Lacertidae, *G. galloti* and *L. schreiberi.*

2) The infection by *Schellackia* was negatively related with the brightness of a ventral blue ornament in males of *S. occidentalis bocourtii*

3) The yellow ornament of the forelimbs in the females of *S. occidentalis bocourtii* infected by *Acroeimeria* was brighter than in infected males. However, there were no sexual differences when uninfected individuals were compared. In spite of that, the brightness of the forelimb was negatively related with the body condition in either sex.

Conclusions

1) The genera *Schellackia* and *Lankesterella* have independent evolutionary origins, and thus, the family Lankesterellidae has not a monophyletic origin

2) The genus *Schellackia* is more diverse and host specific than it was previously known. Indeed, different host lacertid genera from the Iberian Peninsula did not share parasite haplotypes even though some of these lacertid species are sympatric.

3) *Isospora*-like parasites isolated from reptiles are not closely related to *Isospora*-like parasites from birds or mammals. They may be a completely new genus of coccidia.

4) The genus *Caryospora* has not a monophyletic origin. This was evidenced when we characterized an isolate from lizards and it was related closer to genus *Lankesterella* than to *Caryospora* parasites found in mice.

5) Parasites found in reptiles with *Eimeria*-like oocysts form a monophyletic clade. In addition, phylogenetic analyses validate the genera *Acroeimeria* and *Choleoeimeria* previously proposed by Paperna and Landsberg (1989) based on morphologic characteristics of the oocyst stage.

6) The relations found between the blue coloration with either the presence or the load of endoparasites in different host parasites systems are compatible with a higher deposition of eumelanin in the skin of the lizards. Given that high oxidant conditions are required for the synthesis of eumelanin, UV-blue or blue signals are likely to be related with the individual ability to cope with oxidative balance similarly to other vertebrate systems that also show melanin-based traits.

7) Yellow ornaments can be affected by either chronic (endoparasites) or acute and seasonal infections (ectoparasites).

8) In host species where both sexes show similar sexual ornaments, the phenotypic response to parasitic infections can be in opposite direction.

9) In dimorphic species, individuals bearing typical characteristics of the other sex are handicapped. This is the case of "bearded ladies", meaning females with typical male-like traits. For example, females of the American lizard, *Sceloporus occidentalis bocourtii*, and the Canarian lizard, *Gallotia galloti palmae* were in better body condition or were less often parasitized when they showed typical female-like traits. In turn, males with more conspicuous color traits typical of dominant males reflect better individual quality in line with a Zahavi's handicap-like mechanism.

INTRODUCTION

The current number of described species in the world is established around 1.3 million (Costello et al., 2012). However, considering the number of new described species per year, predictive models raise the number of existing species up to 2.1 million (Chapman, 2009; Costello et al., 2012). To this constant and increasing rate of newly described species (Costello et al., 2012), we need to add the plausible existence of at least one specific species of parasite for each existing species. Moreover, some of the species of parasites possess, additionally, their own species of hiperparasites. Parasites evolve different strategies to infect appropriate hosts in which they can undergo their life cycle extracting the appropriate resources that they need to stay alive, reproduce and spread. These strategies do not necessary evolve in the same way. Indeed, this different evolution of parasitic strategies resulted in an immense diversity of parasite-parasite and host-parasite interactions (e.g. Bush et al., 2001). These biological strategies or adaptive changes in the parasites can go from the development of resistant structures evolved to allow the parasite to last in the surrounding environment until the encounter with a suitable host (e.g. Belli et al., 2006), passing through the development of attaching parts to get stack to edible items or even be transmitted in the viscera of an intermediate host when this last is eaten (e.g. Matuschka and Mehlhorn, 1984; Morsy et al., 2011). Other strategies include the use of other organisms to be carried into the host track (e.g. Reichenow, 1920a; Haklová-Kočíková et al., 2014), or more sophisticated evolutionary relationships where the parasite needs to pass through more than one organism to accomplish its entire life cycle (e.g. Otranto and Traversa, 2002; Marquardt et al., 2000). However, the most complex relationships involve parasites that alter the host behaviour to increase its own fitness (Moore, 2002a, b). When these strategies increase the fitness of the individual parasite over other individuals of the same parasite species with different strategies, the genes responsible of the successful characters are inherited by the following generation of parasites and, generation after generation increase their frequency in the population by a process of natural selection (Darwin, 1859).

The current accepted number of reptile species in the world is between 6.300 and 8.734 (Chapman, 2009). Given the high specificity that most parasites reach due to co-evolutionary processes (Adamson and Caira, 1994), it would be expected at least the same number of species of lizard parasites. Among the parasites found in lizards, those belonging to phylum Apicomplexa are the most diverse. Probably, the first reported apicomplexan parasite was in 1674 by Leeuwenhoek. It was a parasite found in the bile of a rabbit. However, it was not until 150 years later when this apicomplexan parasite was described as *Eimeria stiedai* (Lindemann 1896) Kisskalt & Hartmann 1907. Since then, about 5.000 species of Apicomplexa have been described. But, what are the Apicomplexa? This name refers to a phylum of parasitic protozoa with an apical complex used by the parasite cell to actively enter the host cell by degrading its cell membrane

(Periz et al., 2007). Within the phylum Apicomplexa, approximately one third of the existing species belongs to the suborder Eimeriorina within the order Eucoccidiorida Léger and Duboscq 1910 (Figure 1).

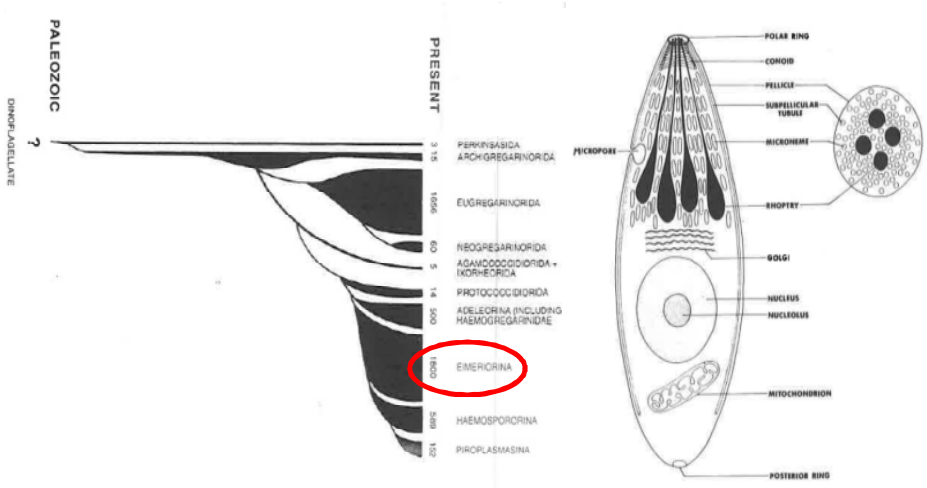

Figure 1. The phyllum Apicomplexa is an ancient group of parasitic organisms that are believed to evolve from Dinoflagelates. All members of the Apicomplexa share the presence of the apical complex at the anterior end of certain stages, commonly sporozoites, merozoites and gammonts. Evolutionary tree and line drawing are from Perkins et al., 2000.

The biological characteristic that all the representatives of the order Eucoccidiorida share is the presence of a generally fixed number of merozoite generations (merogony), and the presence of gamogony and sporogony in their life cycles (Figure 2).

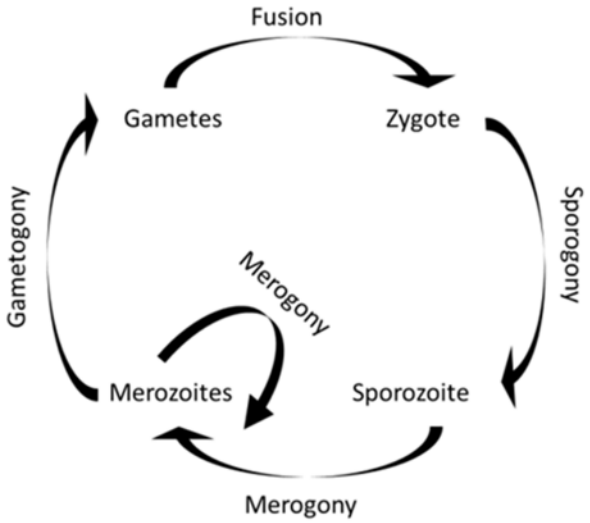

Figure 2. General life cycle of parasites belonging to order *Eucoccidiorida*. The number of merogonic cycles is generally determined within this order.

Coccidia: A model to understand Evolution

Among all the groups of parasites with veterinary and human impact, parasites within the phylum Apicomplexa are one of the most spread and less known groups of parasites although with some important exceptions. The Haemosporidia are apicomplexan protozoa that received major attention since this subclass contains genera related with malaria (*Plasmodium* Marchiafava & Celli 1885, *Leucocytozoon* Sambon 1908 and *Haemoproteus* Kruse 1890). In fact, the discovery of the causative agent of the malaria increased the efforts to study other Apicomplexa (Perkins et al., 2000). Other genera within Apicomplexa that were widely studied are parasites in the genus *Toxoplasma* Nicolle & Manceaux 1909 (Apicomplexa: Sarcocystidae) which may provoke malformation to the fetus during development; and parasites in the genus *Cryptosporidium* Tyzzer 1907 (Apicomplexa: Cryptosporidiidae) which is responsible for chronic enteritis and its virulence is associated with the immunosuppressive status of the host. In the last 50 years we started to understand some aspects of the biology, ecology, systematics and pathology related to infections caused by Apicomplexa. For example, the incidence of some coccidiosis of veterinarian concern stimulated studies on the virulence, genetics, life cycle, infectivity and immunobiology of parasites of the genera *Eimeria* Schneider 1875 and *Sarcocystis* Lankester 1882 (see Dubey, 1976; Allen and Fetterer, 2002). However, despite the high diversity of strategies of infection by parasites found in reptiles and their high specific diversity, the knowledge about the parasites of the Reptilia is scant especially in comparison with other groups of vertebrate hosts. In fact, only in terms of protozoan infections, probably more than 50% of existing species remain unknown (Foissner, 2006).

The present dissertation is focused on coccidian parasites (Apicomplexa: Eucoccidiorida) that infect lizards in different parts of the world. The order Eucoccidiorida is constituted by two suborders: Eimeriorina Léger 1911 and Adeleorina Léger 1911. The host groups that these parasites infect are very diverse and, even in heteroxenous life cycles the intermediate hosts can be vertebrate or invertebrate. The parasites within the Eimeriorina are genera such as *Isospora* Schneider 1881, *Eimeria*, *Caryospora* Léger 1904, *Cyclospora* Schneider 1881, *Schellackia* Reichenow 1919, *Lankesterella* Labbé 1899 and *Sarcocystis*, all of them found in lizards. Parasites within these genera show different ways of infection and a high diversity. In particular, more than 200 species of strictly intestinal coccidia were described parasitizing lizards in the world. In addition, around a hundred more intestinal coccidia species were reported from lizards and remain to be described (see Duszynski, Upton and Couch, 2008). These parasites were classified in the genera *Eimeria* (s. l.), *Isospora*, *Caryospora*, and *Cyclospora*. All these genera of coccidia with strict intestinal cycle undergo their entire life cycle in the reptile host and are transmitted without the aid of any vector (e. g. Barnard and Upton, 1994; Upton, 2000). However, heteroxenous facultative cycles are known for some of these parasites (i.e. *Caryospora*) that may

undergo the entire cycle in viscera out of the intestine and they are transmitted by ingestion of the host (Upton et al., 1986).

The common characteristic to all these genera of parasites is the development of a hard structure of resistance (oocyst) that contains eight infective stages of the parasite (sporozoites). In this sense, the taxonomy of this group had methodological limitations since the 98% of the newly described species were based on the number of sporocysts in the exogenous oocyst (Figure 3; see Duszynski and Wilber, 1997; Ghimire, 2010). Nevertheless, in some groups the oocyst presents endogenous development and has soft walls that break to release the infective stages of the parasite into the host's body. In coccidia with exogenous oocyst, this one lasts in the environment until it is swallowed by the next host.

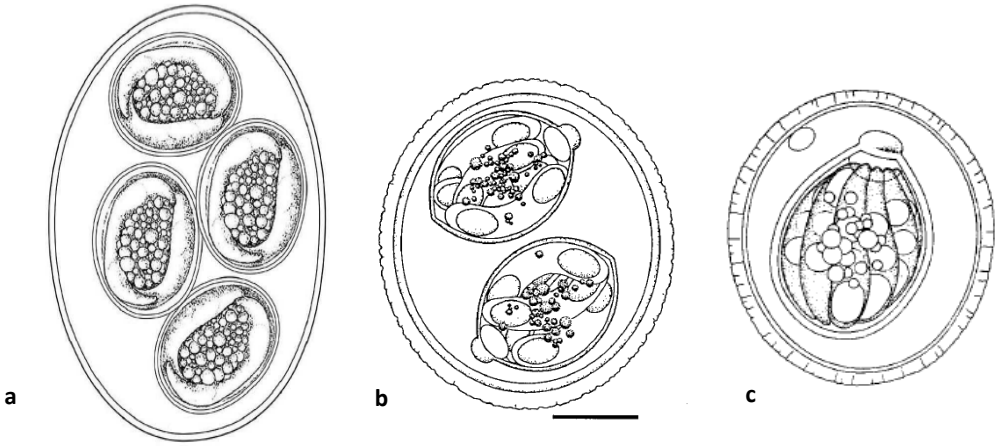

Figure 3. Exogenous oocysts of intestinal coccidia of reptiles. (**a**) *Eimeria* sensu lato; (**b**) *Isospora*; and (**c**) *Caryospora*. All of them contain eight sporozoites which are the infective stages. Line drawings from Upton et al., 1986; Modrý et al., 2001; Al-Quraishy, 2011.

The suborder Eimeriorina groups parasites that may be homoxenous, heteroxenous, facultatively homoxenous, or facultatively heteroxenous. Species develop in vertebrates or invertebrates, and some species alternates both types of host (Upton, 2000). Macrogametocytes and microgametocytes develop independently, and microgametocytes produce many microgametes. Sporozoites develop within environmental resistant oocysts of hard-shelled walls or, in some cases, into soft-shelled endogenous oocysts (Figure 4). The taxonomy of this group is poorly known, due in part to taxonomic methods that neglected the use of microphotographs or type specimens (Upton, 2000). The implementation of molecular techniques and the creation of databases for these organisms (e.g. Duszynski et al., 2008) are improving the systematics of the group.

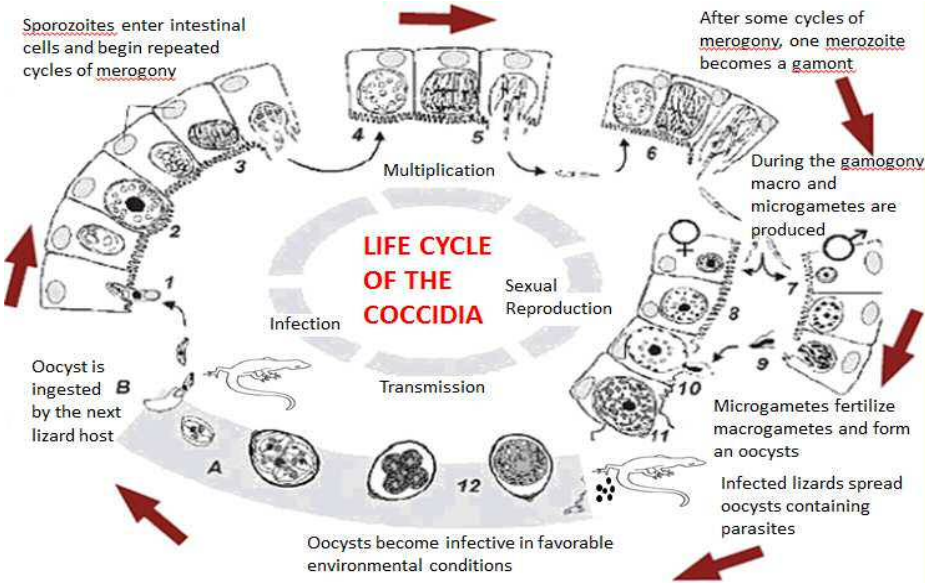

Figure 4. General life cycle of the suborder Eimeriorina in a hypothetical host. Line drawings adapted from http://www.thepoultrysite.com/

Although the infection by intestinal coccidia was related with pathologies such as listlessness, anorexia, weight loss, regurgitation, and enteritis (Barnard and Upton, 1994), few works focused in the taxonomy of the Eucoccidiorida found in reptiles. This fact was also because the relationships among the different coccidia species were hard to disentangle based solely in the characters of the few life stages that were known for most of the species. One striking effort to contribute to the taxonomy of this group was Paperna and Landsberg (1989). In this study, the authors proposed the existence of a reptile-specific lineage of parasites with sporocysts distribution similar to those of parasites within the genus *Eimeria* that were known for other host groups. They claimed that the morphology of the exogenous oocyst was associated to the place in the reptile's intestine where the coccidian parasite underwent its endogenous development (Figure 5). In this sense, they suggested the generic name *Choleoeimeria* Paperna and Landsberg 1989 for parasites with endogenous development in the gall bladder of lizards and that had a ratio between the width and the height of the oocyst above 1.24; whereas *Acroeimeria* Paperna and Landsberg 1989 was proposed for *Eimeria*-like parasites that underwent their oocyst development in the intestine surface with width/height ratios between 1 and 1.24. However, the validity of these taxa has been controversial (e.g. Asmundsson et al., 2006) and despite morphological (Lainson and Paperna, 1999a; Paperna, 2007) and molecular (Jirků et al., 2002) evidences showing the evolutionary peculiarities of the eimerian parasites found in reptiles the genera *Choleoeimeria* and *Acroeimeria* remained neglected by some authors. The implementation of molecular tools for the study of protozoan parasites (Escalante and Ayala, 1995) can help to solve these questions. However, so far only two sequences of *Eimeria*-like parasites found in reptiles had been included

in the phylogeny of the Eimeriorina (Jirků et al., 2009). Although this study supported an independent evolution of the coccidia found in reptiles, whether the morphology of the exogenous oocyst was related with the phylogenetic affinities within this reptile-specific clade remained unknown.

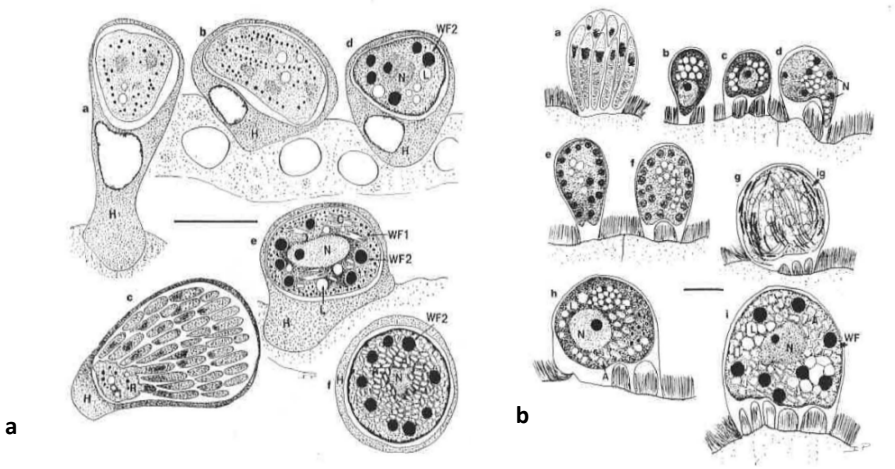

Figure 5. Endogenous development of the *Eimeria*-like parasites that infect reptiles. (**a**) Oocyst of *Choleoemeira* parasite developing in the gall-bladder; and (**b**) endogenous development of *Acroeimeria* parasite in the intestine surface both from gecko host species. Line drawings from Paperna and Landsberg, 1989.

Similarly, the genus *Isospora* was defined to classify coccidian which oocysts contained two sporocysts each of them with four sporozoites. Taxonomic criteria highlight the need to base generic names in monophyletic groups (Ghimire, 2010). In this sense, recent investigations demonstrated independent evolutionary origins for parasites within this genus that infects mammals, birds, and frogs. Therefore, these studies proposed to re-erect several former synonyms for the genus *Isospora*. The genus *Atoxoplasma* Garnham 1950 was proposed *pro parte*, for *Isospora*-like parasites that infect passerine birds with both intestinal and hematic stages (Barta et al., 2005; Atkinson et al., 2008). Among the family Sarcocystidae, the genus *Cystoisospora* Frenkel 1977 was proposed for monophyletic *Isospora*-like parasites that infect mammals. In the same family, Modrý et al. (2001a) proposed the re-erection of the genus *Hyaloklossia* Labbé 1896 for *Isospora*-like parasites of frogs. These findings encourage future research to include in phylogenetic analyses *Isospora*-like parasites found in other hosts, such as reptiles, to understand the phylogenetic affinities among these parasites that may specialize in particular host groups.

In addition to the parasites within the Eimeriorina with exogenous oocysts, the parasites classified in the genera *Schellackia* and *Lankesterella* (Lankesterellidae) evolved heteroxenous life cycles with a paratenic host with a crucial role in the transmission of the parasite (Figure 6). Parasites in the genera *Schellackia* and *Lankesterella* undergo their entire life cycle in the reptile

host remaining as dormant stages (hypnozoites) in the tissues of the hematophagus transmitter (generally a mite, a mosquito or a leech) until this last is swallowed by the next lizard host (Upton, 2000; Telford, 2008). However, the intriguing part of this apparently common cycle is the fact that the parasite develops a soft oocyst wall during its development in the *lamina propia* of the enteric tissue (Telford, 2008). After the maturation of the sporozoites, this soft wall brakes and the sporozoites are released in the blood stream of the peripheral capillaries of the vertebrate host were once they penetrate the erythrocytes (or leukocytes) remain inactive until a blood-sucking arthropod or leech swallows and digest the host erythrocyte (Figure 3). At that moment the sporozoite enters the paratenic host epithelium and remains there dormant. So far, no effect has been described in relation to the infection by these parasites.

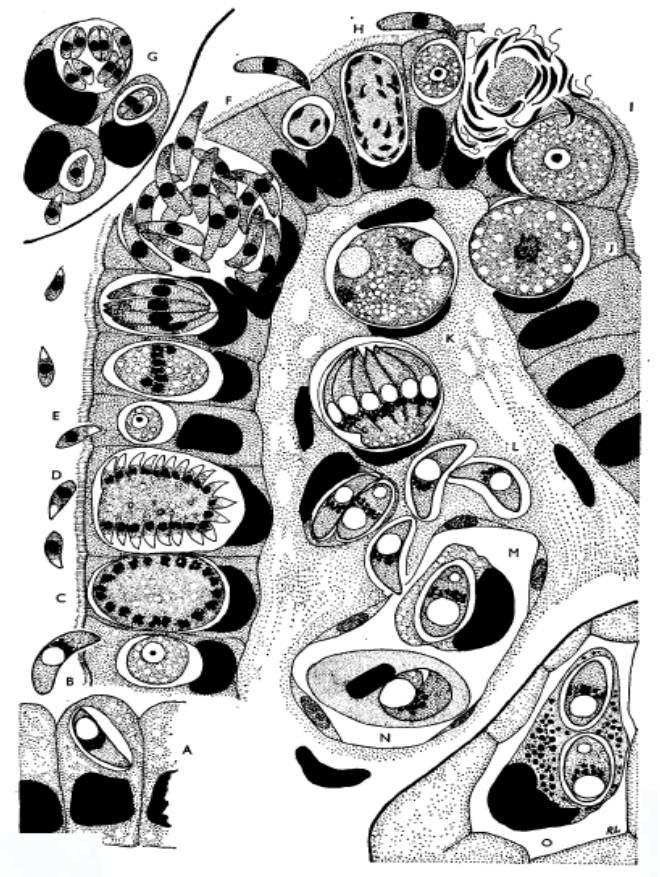

Figure 6. General cycle of hemococcidia of the genera *Schellackia* and *Lankesterella*. From the bottom left to bottom right. (A) hypnozoite in epithelial cell of the arthropod. (B) Sporozoite penetrates epithelial cells of small intestine of lizard. (C, D) Development of microschizonts and micromerozoites. (E, F) Development of macroschizonts and macromerozoites. (G) Asexual division in monocytes and lymphocytes of spleen and liver. (H, I) Development of microgametes and fertilization of macrogametes in epithelial cells of small intestine. (J, K) Entry of fertilized macrogamete into lamina propia and development of oocyst containing eight sporozoites. (L) Liberation of sporozoites from rupturing oocysts. (M, N) Entry of white and red cells of peripheral blood. (O) Infective, diapausing sporozoites in the reticulo-endothelial cells of liver, lung and other viscera. Line drawing from Lainson, Shaw and Ward, 1976.

Morphological studies of hemococcidian parasites in the genera *Lankesterella* and *Schellackia* revealed the presence of electron dense structures or refractile bodies that are commonly found in the ultrastructure of the infective stages of species in the genus *Eimeria* (Figure 7). This result suggested that parasites within these genera were evolutionary close related to other genera in the family Eimeriidae Minchin 1903 (Paperna and Ostrovska, 1989; Klein et al,. 1992; Paperna and Lainson, 1999). In addition, some characteristics of the life cycle of the hemococcidia, *Lankesterella* and *Schellackia*, such as infecting reptiles and amphibians, and the presence of heteroxenous life cycles motivated the classification of these genera within the family Lankesterellidae.

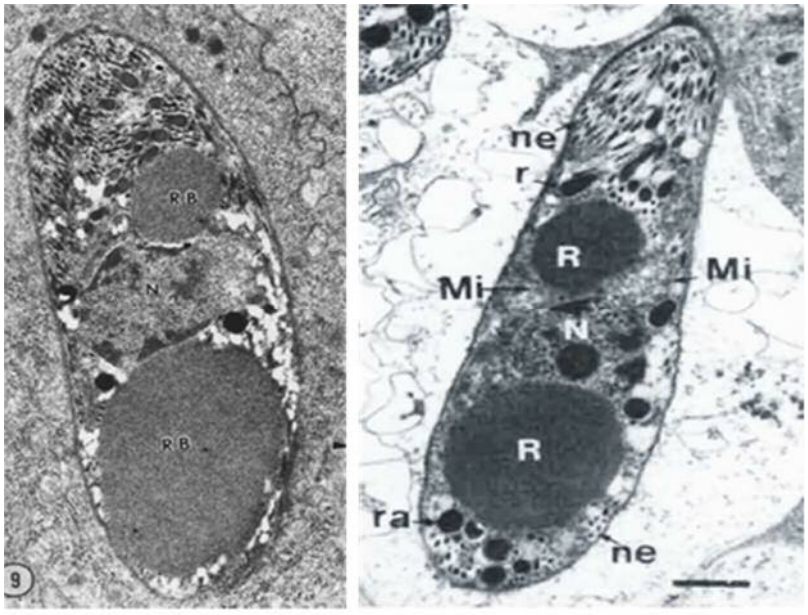

Figure 7. Ultrastructure of a sporozoite of the genus *Eimeria* (left) and the genus *Schellackia* (right). RB and R, are refractile bodies respectively. TEM photographs from Chobotar, Danforth and Entzeroth, 1993; Paperna and Ostrovska, 1989.

Although Grassé (1953) erected the family Schellackiidae to host the genera *Schellackia* and *Tyzzeria* Allen 1936, this family seems to have been ignored in further classifications. Later on, the genus *Tyzzeria* was emended to cover all coccidia species with exogenous oocysts containing naked sporozoites and that infected Anseriformes (Aves). The few species described for lizards (Probert et al., 1988) were later synonymized with *Eimeria*-like species because it was evidenced that type specimens of *Tyzzeria* spp. that infected lizards were mature oocysts of *Eimeria*-like parasites that had released the sporozoites to the oocyst lumen at the moment of their examination (see Paperna and Landsberg, 1989; Ball et al., 1994). In addition, so far no sequence belonging to parasites within the genera *Schellackia* or *Lankesterella* found in reptiles had been included in the phylogeny of the family Eimeriidae to study the evolutionary relationships of these parasites that were even associated with the ancestors of malaria-parasites (Manwell, 1977).

Lankesterellids are found in lizard species around the world in all places inhabited by reptiles (Telford, 2008) evidencing that host-parasite relationships in this group may be old (Manwell, 1977). A long evolutionary relationship is one of the factors influencing parasite specificity (Adamson and Caira, 1994), thus the current number of species in these genera might be increased as long as taxonomic effort increased in these groups.

On the other hand, the Adeleorina found in reptiles are classified in the genera *Hepatozoon* Miller 1908, *Karyolysus* Labbé 1894, and *Haemogregarina* Danilewsky 1885. Although, following the recommendation of Siddall (1995), the species of *Haemogregarina* spp infecting lizards were reclassified in the genus *Hepatozoon* (Smith, 1996). These parasites undergo part of their cycle in the intestinal tissue of the lizard host, but they need a transmitter for infecting a second lizard host (Telford, 2008). In particular, parasites within these genera undergo the asexual reproduction (schizogony or merogony) in the reptile host and the sexual reproduction (gametogony) and posterior sporogony in the vector (more likely a mite, a mosquito, or a tick species) (Smith, 1996; Haklová-Kočíková et al., 2014). However, the lizard host may not be the definitive host. The recent research made by Tomé et al. (2013) finding *Hepatozoon* haplotypes found in lizards in the blood of snakes supported previous references defending that lizards and frogs are intermediate host for *Hepatozoon* species infecting snakes as final vertebrate hosts (Smith, 1996; Telford, 2008).

In the suborder Adeleorina Léger 1911 motile gamonts of either sex are associated in syzygy prior to the formation of functional gametes, fertilization and sporogony (Figure 8). In heteroxenous genera, in opposition to the heteroxenous genera within Eimeriorina, the sporogony usually takes place in the epithelial cells of an invertebrate host and vector (Upton, 2000). There are seven named families of coccidia in this suborder of either homoxenous or heteroxenous life cycles. The genera *Hepatozoon*, *Haemogregarina*, *Hemolivia* Petit, Landau, Baccam & Lainson 1990 and *Karyolysus* which are found in reptiles around the world, possess the higher number of named species within the Adeleorina. Nevertheless, the adeleorine species that parasitize invertebrates are likely to be the most abundant group within this suborder. However, most of these species remain undescribed (Upton, 2000).

In the Iberian Peninsula these genera of parasites with hematic stages are found in lizards usually infecting erythrocytes in peripheral blood (Reichenow, 1920a; Harris et al., 2012; Maia et al., 2012; Martínez-Silvestre and Arribas, 2015). The infection by hematic coccidia in lizards had been related with physiological and behavioural symptoms. In lizards of different taxonomic families and from different parts of the world it has been described a decrease in hemoglobin concentration (Oppliger et al., 1996), an increase in the number of immature red blood cells (Martínez-Silvestre and Arribas, 2015), an increase of oxygen consumption at rest, a reduction in the locomotor speed (Schall, 1986; Oppliger et al., 1996), and an increase in the reproductive

effort (Sorci et al., 1996), all associated to the infection by hematic cocccidia of this suborder. Furthermore, the infection with these types of coccidia affected the showiness of sexual characters (Martín et al., 2008; Molnár et al., 2013) and altered the scape behaviour in lizards (Garrido et al., 2014). However, the relations between blood parasites of reptiles and the phenotypic response measured in the hosts were not always evident (see García-Ramírez et al., 2005; Stuart-Fox et al., 2009; Damas-Moreira et al., 2014).

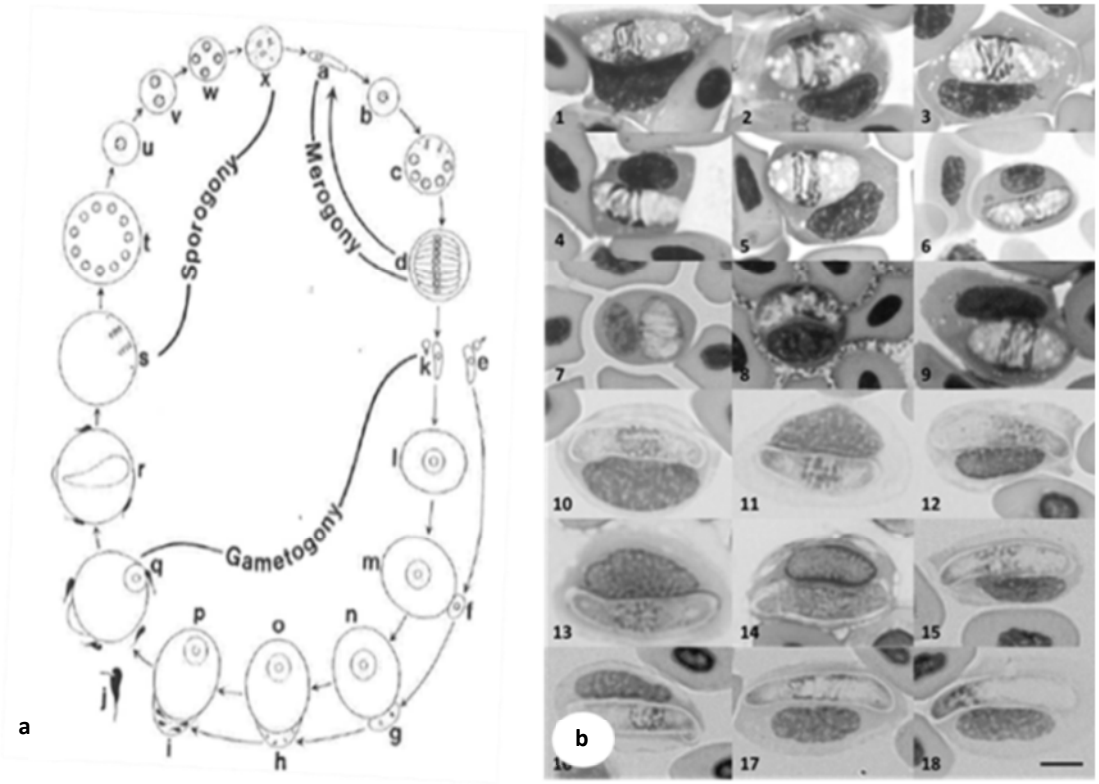

Figure 8. (**a**) General life cycle of an Adeleorina parasite. **a-d**: an infecting sporozoite begins several cycles of merogony within a host cell with production of merozoites that infect new host cells to undergo new merogony; **e-q:** at a specific moment, merozoites develop into gamonts. The development of macrogametocytes and microgametocytes is given in syzygy. **e-j:** microgametocyte formation; **k-q:** microgametocyte formation; **r-x:** sporogony. This step produces the formation of the sporocyst. The result is the formation of naked sporozoites ready to infect the next host. (**b**) Gamonts of an Adeleorine in erythrocytes of an Iberian lacertid (*Lacerta schreiberi*). The gamont distorts the host cell and pushes the host nucleus away from the center of the host cell.

Ectoparasites: vectors, transmitters and blood-suckers

Most of the apicomplexan parasites of heteroxenous life cycles known in lizards are transmitted by blood-sucking arthropods (e.g. Reichenow, 1920b; Smallridge and Bull, 1999; Schall and Smith, 2006; Barta et al., 2012). These ectoparasites are commonly found on the skin of the lizards around the world (Figure 9; Tälleklint-Eisen and Eisen, 1999; García-de La Peña, 2011;

García-Ramírez et al., 2005; Václav et al., 2007) and some on the surface of their respiratory and digestive tract (Fajfer, 2012). However, as commented above, not all the arthropod-borne parasitic diseases are transmitted through the saliva of the vector. Some of them are effectively transmitted when the infected arthropod is swallowed by the next host (e.g. Landau et al., 1972; Bristovetzky and Paperna, 1990; Smith et al., 1994). In this sense, ixodid ticks are known to transmit some pathogenic agents such as bacteria (Dsouli et al., 2006; Majláthová et al., 2008; Ekner et al., 2011; Kubelová et al., 2015) and some Adeleorina (e.g. Landau and Paperna, 1997; Široký et al., 2009), and can inflict severe damage by blood removing (Dunlap and Mathies, 1993).

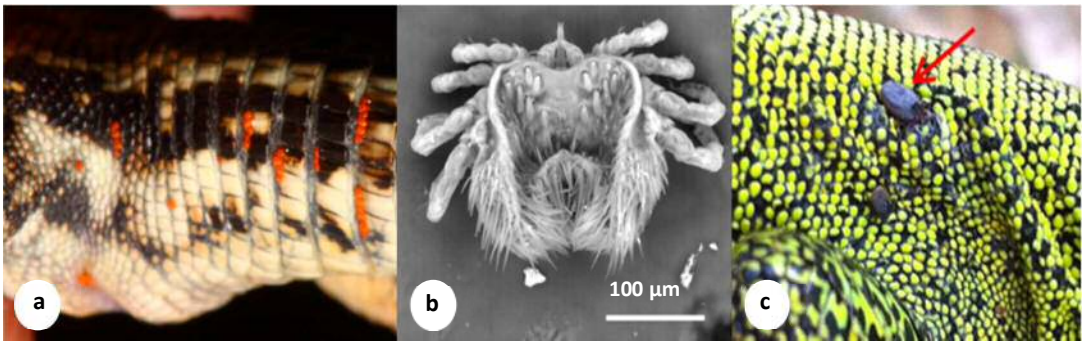

Figure 9. Ectoparasites commonly found attached on lizards around the world. (**a**) mites (Acari: Macronyssidae) attached on *Podarcis muralis* tail (Photo gently given by Javier Ábalos) (**b**) Microphotograph of *Geckobia* mite (Acari: Pterygosomatidae) found on *Tarentola* geckoes (Photo SEM by Juan Hernández-Agüero and Alberto Jorge: MNCN-CSIC), (**c**) *Ixodes ricinus* nymph (Acari: Ixodidae) attached on the back of a male *Lacerta schreiberi*.

Ectoparasite infestations are known to be dependent on environmental conditions and be seasonally-dependent (Tälleklint-Eisen and Eisen, 1999; Schall et al., 2000; Lumbad et al., 2011). This seasonality may be related with the seasonal hormonal balance of their hosts (Salvador et al., 1996; Olsson et al., 2000). Additionally, host susceptibility to these parasites may be genetically dependent (Olsson et al., 2005) and may affect the conspicuousness of the visual ornaments in lizards (Weiss, 2006; Václav et al., 2007). However, in other cases massive infestation by ectoparasites can occur with no apparent effect on the host health (Gomes et al., 2013). Thus, factors such as host-specificity, host individual genetic quality, host hormonal balance or general health status of the host may influence on the pathogenicity and the incidence of ectoparasites (Sorci and Clobert, 1995; Uller and Olsson, 2003; Vilcins et al., 2005; Graham et al., 2012). Overall, ectoparasite infestation consists on acute seasonal symptoms, whereas the pathogenicity associated to endoparasitosis commonly have chronic symptoms and the parasites can be detected in the host over time (Valkiūnas, 2004).

Co-evolving organisms and ecological interactions

Ecological aspects of the biology of the Eucoccidiorida, such as the specific relationships with their hosts, are poorly understood. In this sense, studies on the relationships between coccidian parasites and their hosts are fundamental to understand the co-evolutionary processes that may take place in each specific system. Parasites and hosts interact and co-evolve optimizing their fitness. In co-evolutionary relationships host or parasites may modify features of each other to improve their own fitness (Combes, 2001; Moore, 2002a and 2002b). The arising of such adaptations might be promoted between organisms living in symbiosis for long time (Moya and Peretó, 2011). In this sense, fine adaptive tuning of morphological or ecological characteristics may confer fitness advantages in either the parasite or the host (Pal et al., 2007). An evolutionary theory elegantly explained processes of co-evolution that are constantly taking place among organisms (i.e. Van Valen, 1973). The same year than *The descendant of Man* (Darwin, 1871) was published, the first edition of *Through the looking glass, and what Alice found there* (Carroll, 1871) saw the light. The tale found in that book explained why Alice and the Red Queen had to run twice as fast as they did to stay right in the same place in a running environment. The Red Queen hypothesis (Van Valen, 1973) proposes that events of mutualism, at least on the same trophic level, are of little importance in evolution in comparison to negative interactions. Therefore, the evolution of organism involved in host-parasite relations may be driven by the net result of this interaction (Hamilton 1980, 1990). In this metaphore, the parasites are characterized by the Red Queen and the hosts "are" Alice (Figure 10). Parasites are always, at least, one step forward their hosts in terms of adaptation. This is due to higher mutation rates and shorter times of generation that parasites have in relation to their hosts (Hamilton, 1990; Combes, 2005), which allow them to adapt to a possible event of changing environment, e.g. the host response. In addition to this mutualistic relationship, we shall consider the surrounding changing environment. Thus, considering "Alice" and "the Red Queen" as a whole entity, they run in a changing environment to prevail (Van Valen, 1973).

Paradoxically, parasites cannot go too far forward in the arms race, since the more virulent lines are eliminated from the population by natural selection if they kill the host before being transmitted to the next one (Ewald, 1993). Thus, the evolution of virulence (sensu Read, 1994) may be a self-regulated adaptive process dependent on the rate of transmission success of the parasite (Ewald, 1993). Even though the virulence of the parasites is a self-regulated mechanism, hosts evolve mechanisms of resistance against the transmission of parasitic diseases to avoid the costs on fitness associated to the parasitism (e.g. Merino et al., 2000; Martínez-de la Puente et al., 2010). These mechanisms may be driven by alleles of genetic resistance (Olsson et al., 2000; Rivero-de Aguilar, 2013), that in turn may show phenotypic correlation (Hamilton and Zuk, 1982). In this sense, sexual ornaments displayed during agonistic or sexual interactions may

convey the genetic, hierarchic, and health status of the bearer (Møller et al., 1999). Therefore, parasites may play an important role influencing the communication in animals.

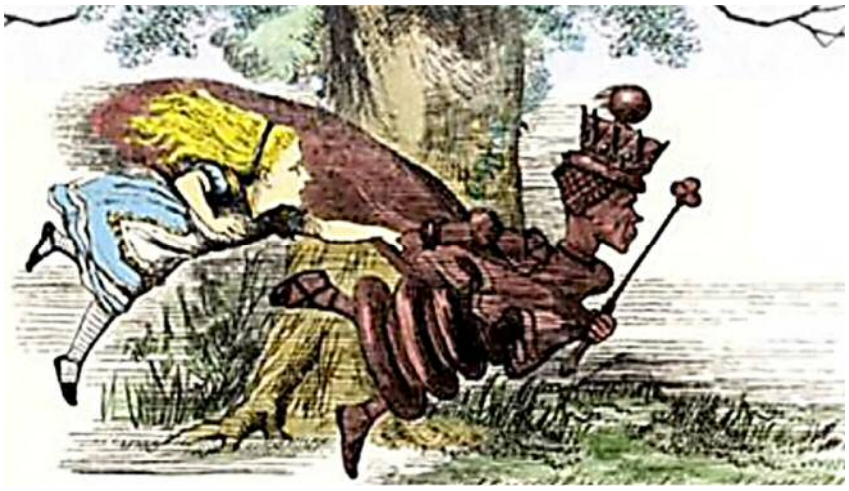

Figure 10. *Now, here, you see, it takes all the running you can do, to keep in the same place. If you want to get somewhere else, you must run at least twice as fast as that!* Through the looking-glass, and what Alice found there (Lewis Carroll, 1871). Illustration made by John Tenniel and extracted from the same book.

Any type of communication (e.g. Wilson and Bossert, 1963; Berger, 1989; Márquez and Bosch, 1995) entails the presence of an emitter of one or multiple messages encoded in signals, and one or more receivers of these signals that will transduce and decode the message (Endler, 1993). However, the interests of the emitter and receiver needs not coincide, even within species (Endler, 1993). For instance, the emitter will produce a signal to increase their chances to find a partner (Bradbury and Vehrencamp, 1998), or to avoid conflicts (Molina-Borja et al., 1998), whereas the receptor will use it to take decisions of whether interact or not with the bearer of the signal (Endler, 1993). Therefore, signals may evolve to favor the fitness of the emitter by manipulating the receiver's decision (Otte, 1974; Dawkins and Krebs, 1978; Guilford and Dawkins, 1991; Wagner, 1992; Endler, 1993). There is a number of factors that can bias the quality of the signals (Endler, 1993), some factors can affect the purity of the signal once it has been sent (see Llusia, 2013), while others can affect the emitter itself (e.g. body condition, body temperature, physiological status) biasing the signal before being emitted. In this sense, organisms living in tight relation with their biological partners might evolve together (Moya and Peretó, 2011), and thus, one of the consequences of this symbiosis is that one or both organisms bias the behaviour of the other one to increase the fitness of one or both of them (Combes, 2001; Moore, 2002a and 2002b).

The Handicap Principle and "a role for parasites" in sexual selection

Zahavi (1975) proposed an evolutionary mechanism that explained the existence of exaggerated or conspicuous traits, usually in the eligible sex. He suggested that these costly traits conveyed to conspecifics the quality of the bearer to stand the handicap associated to the trait (Saino and Møller, 1996). Hamilton and Zuk (1982) proposed a modification of Zahavi's handicap principle (1975). They proposed that chronic infections of parasites handicapped the expression of the sexual signals of their hosts biasing the mating selection and then favoring individuals with the genetic capability to avoid or stand parasitic infections (Møller et al., 1999; Weiss, 2006; Calisi et al., 2008; del Cerro et al., 2010). Thus, species or populations evolving under high pressure of parasites might possess a sophisticated mating system with complex behavioural and ornamented displays that denoted the physiological condition of the actor (Hamilton and Poulin, 1996). Although the effect of the parasites on lizards was not always apparent over the variables measured (García-Ramírez et al., 2005; Stuart-Fox et al., 2009; Damas-Moreira et al., 2014), some studies performed in natural populations of lizards evidenced detrimental effect of parasitism over the infected individuals in either reproductive, ornamentation, or scape behaviour aspects (Oppliger et al., 1996; Václav et al., 2007; Garrido and Pérez-Mellado, 2014). In this sense, parasites related with malaria received major attention in studies involving other vertebrate hosts due in part to its relation with human malaria, and also due to the high incidence of these parasites in natural populations of birds from Europe (e.g. Merino and Potti, 1995; Merino et al., 1997). It is worth to mention that there is not known malaria-like parasites known for European reptiles (Telford, 2008) and the only malaria-related parasite for a lizard species with distribution in Europe is *Haemocystidium tarentolae* (Parrot 1927) Paperna & Landau 1991 described infecting *Tarentola mauritanica deserti* from Algeria (Telford, 2008). Malaria-related parasites have highly specific affinities with their definitive invertebrate hosts (Martínez-de la Puente et al., 2011). In this sense, the American genus *Lutzomyia* (Diptera: Psychodidae) and the species *Culex erraticus* (Diptera: Culicidae) are the known vector for parasites of the genus *Plasmodium* (Apicomplexa: Haemosporidia) infecting lizard hosts in America (Telford, 2008; Fricke et al., 2010; Schall, 2011). In Africa, only indigenous species of haematophagus diptera of the genera *Aedes*, *Culicoides* and *Chrysops* are vectors of *Plasmodium* and related malaria-like parasites in lizards (Telford, 2008). Thus, the restricted distribution of the vectors may limit the presence of haemosporidia parasites in European reptiles. Nonetheless, most of the life cycles of the *Plasmodium* species described for lizard hosts in America, Africa, Asia, and Australasia remain unknown (see Telford, 2008). In this sense, studies on the ecology and the incidence of malaria parasites in reptiles only could be done in some places of the United States where these parasites of reptiles were present and prevalent enough to gather a minimum number of infected individuals to perform consistent studies (e.g. Schall, 1990; Dunlap and Mathies, 1993; Dunlap and Schall,

1995; Paranjpe et al., 2014). In Europe hence, the study of host-parasite relationships and the effect of hemoparasitic diseases in lizards has been restricted to parasites within Adeleorina (Sorci and Clobert, 1995; Sorci et al., 1996; Oppliger et al., 1996; Veiga et al., 1998; Amo et al., 2005a, b, c; García-Ramírez et al., 2005; Foronda et al., 2007; Martín et al., 2008; Stuart-Fox et al., 2009; Harris et al., 2012; Maia et al., 2012; Molnár et al., 2013; Damas-Moreira et al., 2014; Garrido and Pérez-Mellado, 2014; Martínez-Silvestre and Arribas, 2015). However, there is no specific studies on the effects of parasites within Eimeriorina on natural populations of host lizards and then the effect of these parasites remains unknown (Telford, 2008). To my knowledge, only one study explored the effects of *Schellackia* (Eimeriorina) parasites over the ecology of lizards, and it was performed in thermal ecology of the common side-blotched lizards from North America (Paranjpe et al., 2014).

Coloured traits play a key role in sexual recognition and mating access being fundamental in the gene flow of natural populations (Macedonia et al., 2000; Thorpe and Richard, 2001; Leal and Fleishman, 2004; Molina-Borja et al., 2006). Studying environmental factors influencing the expression and conspicuity of these sexual signals is important to understand variables driving the evolution of natural populations. The conspicuousness of colour traits of vertebrates may depend on the combination of both structures and differential allocation of pigments in the dermal chromatophores (Figure 11; Shawkey et al., 2003; Grether et al., 2004; Senar, 2004; Adachi et al., 2005; Olsson et al., 2013) that may be influenced by both genetic and environmental factors (Bajer et al., 2012; Langkilde and Boronow, 2012; Olsson et al., 2013; McLean et al., 2015).

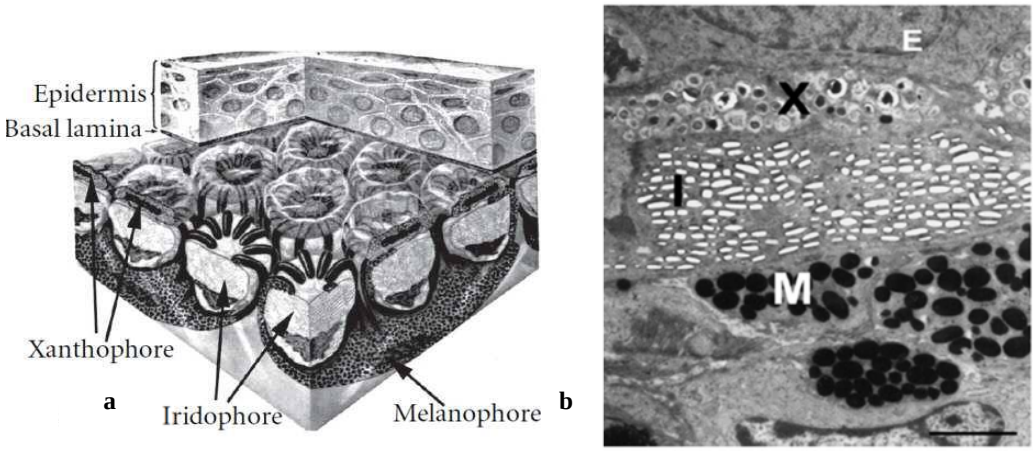

Figure 11. (**a**) Ultrastructure of a lizard skin. Line drawing from Thibaudeau and Altig, 2012. (**b**) The typical structure of the skin of lizards contains melanophores (**M**) (melanin), iridophores (**I**) (platelets of guanine), and xantophores (**X**) (carotenoids and/or pteridines). (**E** epidermal layer). Scale bar= 2 µm. Microphotograph from Kuriyama et al., 2006.

Particularly in lizards, visual ornaments typically involve the deposition of molecules in the skin that may be or may be not synthetize *de novo* in the body of the organism (e.g. Saenko et al., 2013). The first ones are pteridines and melanins, which are synthetized in the body. Pteridines are known for lizards in the American families Polychrotidae (Steffen and MacGraw, 2007) and Phrynosomatidae (Morrison et al., 1995; Weiss et al., 2012; Haisten et al., 2015), and from African Gekkonidae and Chamaeleonidae (Saenko et al., 2013; Grbic et al., 2015) producing red coloured patches (Grbic et al., 2015). Other pigments involved in ornamentation of the skin of lizards are obtained from the diet instead. Such is the case of carotenoids (Olson and Owens, 1998) which modulate immune functions in the body when they are not allocated into the skin (McGraw and Ardia, 2003; Watzl et al., 2003 but see Kopena et al., 2014) and produce yellow, orange and red colour patches when they are allocated in the skin (e.g. San-José et al., 2013). This pigments that are deposited in the xantophores in the skin of lizards, can be differentially removed from the skin of voucher lizards using amonium hidroxid for dissolving pteridines (Figure 12), or acetone for washing carotenoids (Fitze et al., 2009; Saenko et al., 2013; Grbic et al., 2015).

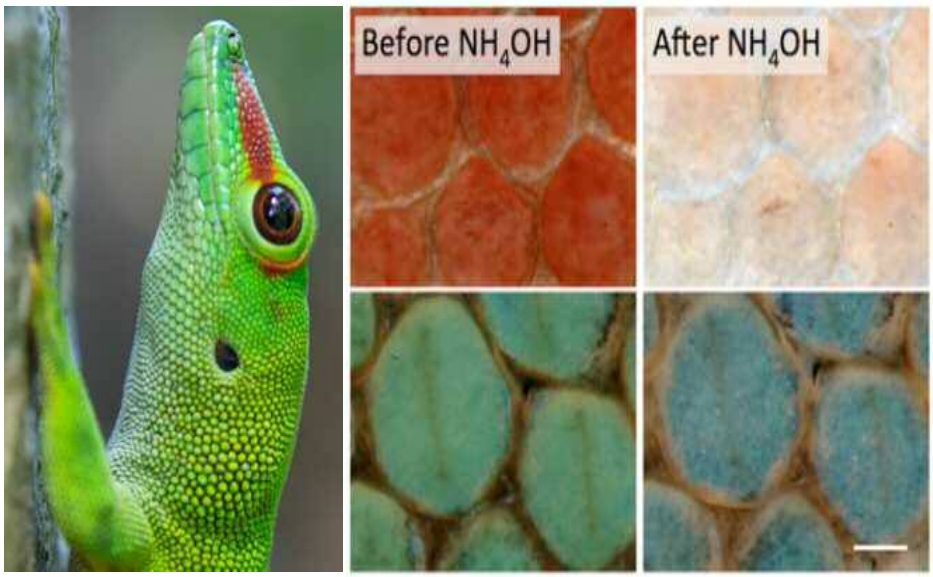

Figure 12. Lizard skin from *Phelsuma* geckoes treated with nitric hidroxid which differentially washes pteridines and leaves the remaining pigments and carotenoids untouched. Pictures from Saenko et al., 2013.

Black, gray, brownish and some yellowish ornaments in different vertebrate groups are the result of the deposition of melanins in chromatophores of the skin (Senar, 2004; Adachi et al., 2005; Roulin et al., 2007; Vroonen et al., 2013). Melanin deposition in the melanophores is the

result of the endogenous metabolism of the organism under specific physiologic conditions that can be costly to the individual (Ducrest et al., 2008; Galván and Alonso-Álvarez, 2009). Melanin concentration has been related with the individual susceptibility to oxidative stress (Galván and Alonso-Álvarez, 2008, 2009). The synthesis of eumelanin, which is the main type of melanin in reptile skin (Ito and Wakamatsu, 2003 but see Roulin et al., 2013), is promoted under high oxidant condition in the melanophores in the basal layers of the dermis (Galván and Solano, 2015). Eumelanin-based ornaments may be conveying the bearer's ability to stand high oxidative stress by recirculating alternative antioxidants than glutathione (e.g. carotenoids) (Galván and Alonso-Álvarez, 2008). Melanic polymorphism, such as black and blue morphs, often occurs in insular lizard populations as adaptation to the high ultraviolet radiation in insular habitats (Pérez i de Lanuza and Font, 2010; Raia et al., 2010). Additionally, other vertebrates, such as birds, show melanin-based traits that result from the combination of pheo- and eumelanin concentration (Senar, 2004). The economy of the melanin in bird ornamentation is related with oxidative levels and the synthesis of one type of melanin is favoured in detriment of the other one (Galván and Solano, 2009). Indeed, studies on birds evidenced the honesty of melanin-based traits in relation with oxidative balance in the body (Roulin et al., 2007; Galván and Alonso-Álvarez, 2008; Almasi et al., 2012). Thus, these patches may signal individual quality in lizards (Vroonen et al., 2013) and thus, they are susceptible to intra- o intersexual selection (Bajer et al., 2010; Olsson et al., 2011). Although some studies explored melanin-based traits in lizards as signals of quality (Vroonen et al., 2013; Molnár et al., 2013; Pérez i de Lanuza et al., 2014), physiological processes underlying the role of melanin-based traits as quality signals was studied in depth in other vertebrates. In this sense, one study evaluated the effect of the experimental infection in moulting birds with endoparasites of the genus *Isospora*. They tested the effect of the infection on the expression of two different coloured traits (yellow and black) (McGraw and Hill, 2000). In this study they found and effect over the carotenoid-based trait but failed to find any relation between parasitosis and the melanin-based trait suggesting that physiological infection may not be equal in different coloured patches or, alternatively, the tested parasite implies detrimental effects on the metabolism of only one of the studied pigments. In addition, previous studies failed finding effects of the coccidial infection on a sexually monochromatic melanin-based trait in the house finch likely because the studied trait is not under sexual selection pressure in this species (Hill and Brawner, 1998). However, melanin-based traits production and maintenance may be costly (Jacquin et al., 2011; Mougeot et al., 2012) and investigation on the effects of parasitemia and melanin-based traits will require further attention.

Parasites cause tissue damage (Chen et al., 2012), hormonal alterations (Dunlap and Schall, 1995), and promote oxidative imbalance (Becker et al., 2004; López-Arrabé et al., 2015). Therefore, parasitic diseases may contribute to imbalance homeostasis in the host's organism

depleting the total availability of endogenous antioxidants (Atamna et al., 1997) and inducing re-allocation of other antioxidants, such as carotenoids (Goodwin, 1986). Thus, carotenoid availability may trade-off between antioxidant function and visual ornamentation (Alonso-Álvarez et al., 2007). Those individuals with genetic competence to avoid or resist the infection by parasites would signal it through the conspicuousness of their ornaments and/or displays (Hamilton and Zuk, 1982). Hence, these ornaments may honestly convey the bearer's health biasing the election of potential mates that may minimize the risk of infection (e.g. Freeland, 1976 in Møller et al., 1992), may bequeath good quality genes of resistance to infection onto the offspring (Hamilton and Zuk, 1982; Hamilton, 1990), may select partners with good body conditions that will be able to take care of the progeny (e.g. in birds: Møller et al., 1992), or may increase the fitness of the offspring by transmitting genes of attractiveness (Weatherhead and Robertson, 1979). Thus, sexual selection *per se* and the existence of sexual reproduction may allow the host to keep adapting to the rapidly changing characteristics of the parasites (Hamilton, 1990).

Hamilton and Zuk's hypothesis (1982) was previously tested in lizards. However, typically the score of colour patterns in lizards were performed subjectively from one observer (e.g. Schall, 1986; Ressell and Schall, 1989; Lefcourt and Blastein, 1991). The present investigation implemented spectrophotometric tools to objectively score colours in lizards (e.g. Font and Molina-Borja, 2004; Martín et al., 2008; Martín and López, 2009; Molnár et al., 2013; Bohórquez-Alonso and Molina-Borja, 2014; Pérez i de Lanuza et al., 2014). These tools in combination with previous methods to analyze colour spectrums (see Endler, 1990) allow to quantify colour in visual traits of lizards. In addition, we studied three different host-parasite systems because, as commented above, the diversity of parasites in lizard hosts may be higher than thought, as evidenced by taxonomic studies that describe new parasite species when a parasite is found in a new host (e.g. Modrý et al., 2001b; Asmundsson et al., 2006; Daszak et al., 2009). Therefore, specific relations may occur in different host-parasite systems.

MAIN OBJETIVES

In the present thesis we studied evolutionary relationships among different parasites of reptiles of the suborder Eimeriorina. In addition, the effect of different parasitic diseases caused by parasites in the Eimeriorina and Adeleorina, nematodes and ectoparasites were studied in three different host-parasite systems. All these studies had the following objectives.

1. Identify and characterize the hemoparasites of *Lacerta schreiberi* and *Podarcis* cf. *hispanicus* using molecular tools.

2. Study the phylogenetic relationships of the genera *Schellackia* and *Lankesterella* to contextualize them within the evolution of the Eimeriorina.

3. Explore the molecular diversity and specificity of parasites within the genera *Schellackia* that infect the Iberian lizards in the family Lacertidae.

4. Contextualize in a phylogenetic framework intestinal parasites within the genus *Isospora* that infect indigenous lizards from different parts of the world.

5. Contribute with phylogenetic support to the systematics of the *Eimeria*-like parasites (*Acroeimeria* and *Choleoeimeria*) that infect indigenous lizards from different parts of the world.

6. Provide information of the effect on visual UV-blue ornaments of infection by hematic parasites of the genus *Karyolysus* in a host insular species of lizard (*Gallotia galloti*) with visual UV-blue ornaments.

7. Provide information of the effect on the conspicuousness of the blue and yellow ornaments on males infected by hematic parasites of the genus *Schellackia* in two different host species: *Sceloporus occidentalis bocourtii* (Phrynosomatidae) and *Lacerta schreiberi* (Lacertidae).

8. Provide information on the phenotypic response to infections by parasites of the genus *Acroeimeria* on the blue and yellow ornaments in a phrynosomatid species (*S. occidentalis bocourtii*) where both the males and the females showed visual ornaments.

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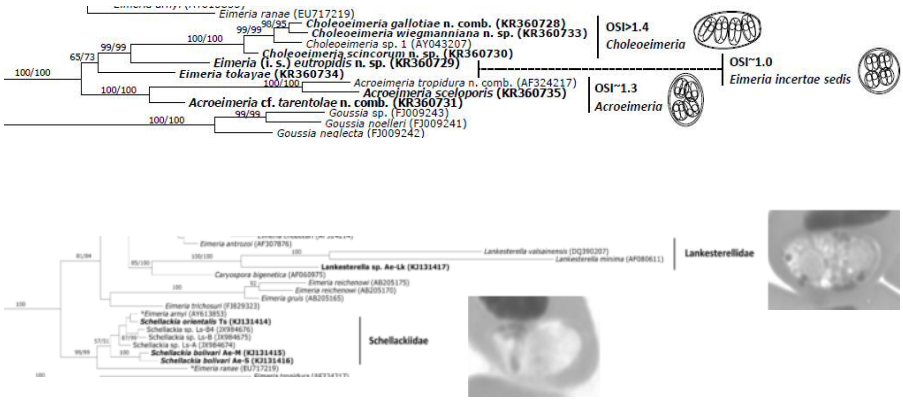
CHAPTER I



Evolutionary relationships of coccidia infecting lizards

Following these lines five studies on the phylogenetic relationships among coccidian parasites that infect reptiles are presented. These studies evidence the different solutions found by coccidian parasites along the evolution of the Eimeriorina to succeed in the transmission and infection of different niches in the physiognomy of the reptilian hosts. In addition, no cross-infections among genera of lacertid hosts were found across the Iberian Peninsula or the pet stores where some of the parasites were sampled suggesting a high degree of parasitic specificity. Furthermore, we evidenced the need of combine molecular and morphological methods for the quantification and the correct identification of the parasitic infection in lizards.

PHYLOGENETIC ANALYSIS BASED ON *18S* rRNA GENE SEQUENCES OF *SCHELLACKIA* PARASITES (APICOMPLEXA: LANKESTERELLIDAE) REVEALS THEIR CLOSE RELATIONSHIP TO THE GENUS *EIMERIA*

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Abstract

In the present study we detected *Schellackia* hemoparasites infecting the blood cells of *Lacerta schreiberi* and *Podarcis hispanica**, two species of lacertid lizards from central Spain. The parasite morphometry, the presence of refractile body, the type of infected blood cells, the kind of host species, and the lack of oocysts in the fecal samples clearly indicated these blood parasites belong to the genus *Schellackia*. Until now, the species of this genus have never been genetically characterized and its taxonomical position under the Lankesterellidae family is based on the lack of the exogenous oocyst stage. However, the phylogenetic analysis performed on the basis of the *18S* rRNA gene sequence revealed that species of the genus *Schellackia* are clustered with *Eimeria* species isolated from a snake and an amphibian species but not with *Lankesterella* species. The phylogenetic analysis reject that both genera share a recent common ancestor. Based on these results we suggest a revision of the taxonomic status of the family Lankesterellidae.

Keywords: Eimeriidae, haplotypes, hemococcidia, *Lacerta schreiberi*, lizard, phylogeny, *Podarcis hispanica*, taxonomy

***Nota bene:** In 2014 Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet redescribed several cryptic species of the *Podarcis hispanicus* complex. The present manuscript of Megía-Palma, Martínez and Merino was published in Parasitology (2013) 140: 1149-1157 before Geniez et al. 2014. Technically *P. hispanica* here is the new variant *P. guadarramae* sensu Geniez et al., 2014. Zootaxa 3794 (1): 001-051.

Introduction

Due to the few works published characterizing at the molecular level apicomplexan parasites from reptiles, it is not rare that relationships of many of these protozoan species were unresolved (Smith, 1996; Tenter et al., 2002; Jirku et al., 2009; Morrison, 2009). In this sense, the hemococcidia group is a paradigmatic example. According to Telford (2008), hemococcidians include three different genera, *Lankesterella* (Labbé, 1899), *Schellackia* (Reichenow, 1919) and *Lainsonia* Landau, 1973, under the Family *Lankesterellidae*, although Upton (2000) considers *Lainsonia* as a synonym of S*chellackia*. Lankesterellids are considered closely related to the intestinal parasites belonging to the Eimeriidae family (Telford, 2008), and parasites of the genus *Lankesterella*, the only genus from the family Lankesterellidae for which molecular data exist to date, falls within the Eimeriidae in recent molecular phylogenies (Barta, 2001; Barta et al., 2001; Jirku et al., 2009; Morrison, 2009; Ghimire, 2010). Biologically, gametogony and sporogony processes are similar in both hemococcidians and intestinal coccidians except in the absence of sporocyst formation in lankesterellids (Telford, 2008). However, in the intestinal coccidians the infective stages are the oocysts expelled in feces whereas in the hemococcidians the sporozoites leave the oocysts at intestinal level, pass to the bloodstream where they penetrate blood cells and then are ingested by an acarine, dipteran or hirudinean hematophagous animals acting as passive vectors (Upton, 2000). At least for saurian hosts, the transmission is finally accomplished by predation of the infected invertebrate (Telford, 2008).

Traditionally the genera *Schellackia* and *Eimeria* have well-demarcated taxonomical boundaries based on their life cycles and their modes of transmission and, therefore, they have been clustered into different families (Lankesterellidae and Eimeriidae). However, there is an increasing consensus that life cycle or host associations may not reflect the evolutionary history within the Apicomplexa (Moore and Willmer, 1997; Barta, 2001). This fact, together with the scarcity of differential phenotypical traits, stimulated the use of molecular phylogenetics based on molecular data to shed light on the relationships within apicomplexan parasites (Barta, 2001; Merino et al., 2006; Jirku et al., 2009; Morrison, 2009). In this sense, recent phylogenetic analyses have shown that the genus *Eimeria* does not form a monophyletic group (Jirku et al., 2009; Morrison, 2009) and the term *Eimeria* sensu lato had been proposed for this group (Jirku et al., 2009). Other authors, highlighting the importance of the use of monophyletic clades in taxonomy, go even farther, suggesting the "phylogenetic destruction" of the genus *Eimeria* due to its paraphyly (see Morrison, 2009).

The life cycle of *Schellackia* lacks exogenous stages (Bristovetzky and Paperna, 1990), so that identification of these parasites relies solely on detection and characterization of endogenous stages. On the other hand, little is known about the morphology of the endogenous stages of most *Eimeria* species apart from the characteristic oocysts released in feces (Upton, 2000; Atkinson et

al., 2008). Although the occurrence of extra-intestinal stages in some species from the genus Eimeria have been previously reported (Mottalei et al., 1992; Carpenter, 1993; Ghimire, 2010 and references therein), these parasitic stages are unknown in more than 98% of all described species (Duszynski and Wilber, 1997; Ghimire, 2010). Interestingly, the infective blood stages of *Schellackia* are morphologically similar to certain extraintestinal stages present in some species of Eimeria (Paperna and Ostrovska, 1989, see discussion below). However, in contrary to the case in *Schellackia*, *Eimeria* parasites have never been detected in blood cells. These data are based on few observations because there are only twelve named species of *Schellackia* (Upton, 2000) and the studies on *Eimeria* genus are mainly based on the analysis of exogenous occysts (Duszynski and Wilber, 1997; Alyousif et al., 2005; Jirku et al., 2009; Ghimire, 2010; Daszak et al., 2011). Other coccidian genera possessing blood stages in their life cycles are *Isospora* and *Atoxoplasma* both isolated from leucocytes of passerine birds (Atkinson et al., 2008).

Although molecular analysis of *Eimeria* from diverse hosts (e.g. mammals, birds, amphibians and reptiles) has been carried out from fecal stages (Honma et al., 2007; Jirku et al., 2009; Power et al., 2009), there has been no molecular analysis of *Schellackia* which is characteristic of lizards. In the present study we describe the morphology of *Schellackia* hemoparasites in lizards from the Iberian Peninsula and, for the first time, carry out molecular phylogenetic analysis.

Material and methods

Lizards sampling

In total, 115 (78 in 2011 and 37 in 2012) Schreiber's green lizards (*Lacerta schreiberi* Bedriaga, 1878) were collected in a deciduous forest in Segovia (Spain) by noosing and hand from early spring to late summer. This is the only period when lizards are available for study because they enter hibernation for the remaining part of the year (Marco, 2011). *Lacerta schreiberi* is a dimorphic midsize lacertid endemic to the Iberian Peninsula (Portugal and Spain) inhabiting humid forests and linked to streams (Marco, 2011). Adult male snout to vent length (SVL) averaged: 96.19 ± 7.59 (80-113) mm, N=42 and adult females SVL averaged 104.04 ± 9.68 (84-123) mm, N=25 in this population in 2011. In addition, 7 *Podarcis hispanica* were captured in the same area. *Podarcis hispanica* is a facultative rock-dweller midsize lacertid lizard with SVL: 38-70 mm in males and SVL: 37-67 mm in females (see Salvador, 1997).

Blood sampling

Blood samples were taken from the ventral vein at the base of the tail (Salkeld and Schwarzkopf, 2005) by puncture using a syringe needle (BD Microlance 3; 23G: 0.6 x 25 mm). The skin around the area of puncture was previously cleaned with ethanol 96% to avoid potential fecal

contamination. Blood was collected with the help of a heparinized capillary tube. Two samples were obtained from each lizard: blood smears were made from one drop of the sample, while the remaining blood was preserved in Whatman FTA Classic Cards (FTA® Classic Card, Cat. No. WB12 0205). The FTA cards were stored in plastic bags with silica gel for later DNA extraction. All blood smears where immediately air dried and later, within the same day, fixed with absolute methanol (Svahn, 1975). At the end of the field season, all blood smears were stained with Giemsa stain (1/10 v/v) for 45 minutes. Slides were examined for hemoparasites following Merino and Potti (1995) and were double-checked in the few cases when we found differences in results between microscopic and molecular analyses (see results). The intensity of infection in the sample was calculated counting the total number of cells infected per 10.000 erythrocytes divided by the number of infected individuals (Stuart-Fox et al., 2010). In the three cases where we obtained intensities of less than 1 parasite per 10000 erythrocytes intensity was considered as 0.5 parasites per 10.000 erythrocytes. The prevalence of infection in the population was calculated as the percentage of individuals infected. Pictures of parasites were taken with an adjustable camera for microscope (Olympus SC30) incorporated to a microscope U-CMAD3 (Olympus, Japan). Length and width of the intracellular parasites were measured with the MB-ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/).

Fecal samples

In 2011, nineteen fecal samples were directly collected into plastic vials (2 ml) from the cloaca of those lizards defecating spontaneously during handling. The feces were stored at -80°C. These samples were exclusively used to perform molecular analysis (see molecular methods). During the field season of 2012, individual lizards were radiotracked by supplying them with small transmitters (BD-2 transmitters, 1.4 g.; Holohil Systems Ltd., Ontario, Canada) allowing us to capture every lizard at least three times during a period of 24 days, thus obtaining different fecal samples from the same individual. At every capture we obtained systematically fecal samples from all individuals by briefly massaging the belly of the lizards and collecting the sample directly from the cloaca as indicated above. Following this method we collected 124 fecal samples from 37 individuals. In this way we increased the chances of detecting coccidian oocysts from individual lizards because shedding is not continuous and depends on several factors (López et al., 2007). In 2012, fecal samples were stored in 2% potassium dichromate for at least 48 h to allow the sporulation of oocysts and thereafter were subjected to concentration by flotation in 15 mL of sugar solution prior to microscopic examination in search of oocysts (Duszynski and Wilber, 1997). We could not obtain fecal samples from *P. hispanica*.

DNA extraction and PCR

We extracted parasite DNA from blood preserved on FTA cards corresponding to lizards captured in 2011 by applying the following protocol: FTA punches were transferred to collection vials with 250 µL of SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH = 8). Immediately, SDS 20% (7 µL) and proteinase K (50 µg) were added to the vials and incubated at 55°C overnight using a thermo-shaker. The next day, ammonium acetate 5 M (250 µL) was added to the vials and incubated for 30 min at room temperature. Subsequently, vials were centrifuged at 13 000 g for 10 min. After removing the pellet, DNA was precipitated with ethanol and re-suspended in sterile water. DNA of the fecal samples was extracted using the UltraClean® Fecal DNA Isolation Kit (Mo Bio Laboratories, Inc).

Due to the lack of previous genetic information for *Schellackia* parasites we first tried partial amplification of the *18S* rRNA gene sequence using primers for other hemococcidians as hep900F (5´ GTC AGA GGT GAA ATT CTT AGA TTT G 3´) / hep1615R (5´ AAA GGG CAG GGA CGT AAT C 3´) or hep50F (5´ GAA ACT GCG AAT GGC TCA TT 3´) /hep1600R (5´ AAA GGG CAG GGA CGT AAT CGG 3´) (see Merino et al., 2006). In order to obtain a larger fragment or to perform internal readings the primers hep600F1 (5´ TCG TAG TTG GAT TTC TGT CG 3´), EIMROD-R (5´ GCA TTT CCC TAT CTC TAG TCG G 3´) and Isosp-R (5´ ATT GCC TCA AAC TTC CTT GC 3´) were designed on the basis of the first sequences obtained. The primer BT-F1 (5´ GGT TGA TCC TGC CAG TAG T3´) was used in the same way (Criado-Fornelio et al., 2003).

To perform a systematic and specific screening of the blood samples, we used the primers hep600F1 / hep1600R (~1000 bp). As the quality of the DNA extracted from fecal samples is lower than that extracted from blood samples, we facilitated the amplification using the primers hep600F1 and Isosp-R which yield a shorter amplicon (800 bp aprox.). PCR reaction volume (20µl) contained between 20 and 100 ng of template DNA, 50 mM KCl, 10 mM TRIS–HCl, 1.5 $MgCl_2$, 0.05 mM of each dNTP, 0.5 M of each primer, and 1.25 U of AmpliTaq Gold 360 (Applied Biosystems, Foster City, Calif.). The reactions were cycled under the following conditions using the Verity thermal cycler (Applied Biosystems): 95°C for 10 min (polymerase activation), 40 cycles at 95°C for 30 s, annealing temperature at 58°C for 30 s, 72°C for 80 s and a final extension at 72°C for 10 min. All amplicons were sequenced to discriminate the haplotypes.

Sequences of *Schellackia* haplotypes were deposited in GenBank under the following accession numbers: haplotype Ls-A: JX984674; haplotype Ls-B: JX984675; haplotype Ph-B4: JX984676.

Phylogenetic analysis

DNA sequences were obtained from the Acacia Website (David Morrison, http://acacia.atspace.eu/Alignments.htm) where Whipps et al. (2012) deposited a nexus file containing most of the Eimeriorina species (454 sequences) which were initially aligned by secondary structure following the model of Gutell et al. (1994). Thereafter, the alignment was refined and manually optimized as indicated by Whipps et al. (2012). To perform the phylogenetic analyses we only used sequences belonging to the families Lankesterellidae and Eimeriidae (194 sequences). In order to decrease redundancy of the alignment, we suppressed all sequences with identity 99%, or higher, using the program JALVIEW (Waterhouse et al. 2009). In addition, three sequences of *Lankesterella* and the three sequences of *Schellackia* were manually aligned on this file. The final alignment contained 67 sequences. Poorly aligned positions and divergent regions of the alignment were suppressed using GBlocks program (Talavera and Castresana, 2007) selecting the following options: "Minimum Number of Sequences for a Flank Position" to 34, "Maximum Number of Contiguous Nonconserved Positions" to 10, "Minimum Length of a Block" to 5, and "Allowed Gap Positions" to "With Half". The GBlocks program suppressed 18% of ambiguous sites. The final alignment (1626 bp) was analyzed using both Bayesian and maximum-likelihood inference. Bayesian inference was performed using the program MrBayes v3.2 (Ronquist and Huelsenbeck, 2003). We used a single partition with the GTR+I+G substitution model. This analysis consisted of 2 runs of 4 chains each, with 6000000 generations per run and a burn-in of 600000 generations (108000 trees for consensus tree). The final standard deviation of the split frequencies was lower than 0.01. Convergence was checked using the Tracer v1.5 software (Rambaut and Drummond 2007). All model parameters were higher than 100. On the other hand, the maximum-likelihood inference was performed using PhyML program (Guindon et al., 2010). The substitution model used was GTR+I+G, the subtree pruning and regrafting (SPR) and the nearest-neighbor interchange (NNI) tree-rearrangements were selected, and the approximate likelihood-ratio test (aLRT) was used to obtain the clade support.

Results

Blood smears

In 2011, the prevalence of *Schellackia* sp. in blood smears was 29.5% (23/78) and the mean intensity per 10000 erythrocytes was 8.9 parasites. All the blood samples that were positive by PCR scanning were also positive by microscopy after checking again three smears found negative in a first check. These three smears showed low parasitemia (<1/10000; see microscopic methods in "Blood sampling" above). The sporozoites of *Schellackia sp.* were always found in erythrocytes but in some parasitized individuals (21.7%, 5 of 23 infected animals) parasites were also detected

in leucocytes as lymphocytes, monocytes, thrombocytes and azurophyles (Fig. 1, E-F and K-O). They were never detected in granulocytes.

The general shape of the sporozoites of *Schellackia sp.* in *L.schreiberi* varied from elongated to rounded. In some cases, a single refractile body can be seen in the cytoplasm. The measures of the sporozoites infecting erythrocytes and leucocytes are shown in Table 1. The nucleus of the sporozoites infecting erythrocytes showed a variety of shapes and placements (see Fig. 1). Forty eight percent of sporozoites showed nucleus touching the parasite membrane in the pointed end of the parasite as two narrow bands one at each side of the parasite (see Fig. 1 I) or occupying a broad area only in one side of the parasite (see Fig.1 B, C and D). The other 52% of the sporozoites showed a band-like nucleus usually situated closer to the broader end of the sporozoite (see Fig.1G, J, K and M). Parasites showed a soft-stained cytoplasm in a 70.5% of the cases (Fig. 1, see G), being darker in the rest of parasites (Fig. 1, see D).

In 2012 we only conducted microscopic examination of blood samples and seventeen out of the thirty seven individuals of *L.schreiberi* sampled (45.9%) were found infected by sporozoites of *Schellackia sp.* The mean intensity was 7.2 parasites /10.000 erythrocytes. Parasites in this year showed similar characteristics to those found in 2011.

In addition, we found one individual of *Podarcis hispanica* (14.28% (1/7)) infected by *Schellackia.* In this case parasites were always found inside erythrocytes. We only find 5 parasites in the whole slide (more than 50.000 cells scanned). The size of these parasitic stages was 5.94 ± 0.73 (5.1-6.77) µm in length and 2.98 ± 0.35 (2.56-3.32) µm in width (N=5) and lack of a visible refractile body in the cytoplasm. They have a pyriform shape, and the nucleus appeared in contact with the cytoplasmic membrane. The cytoplasm is dark stained (see Fig. 1, P-R).

Genetic analysis of blood samples

Overall, 125 blood samples from 78 *Lacerta schreiberi* captured during the year 2011 were analyzed by means of PCR, being 29.5% (23/78) positive for *Schellackia.* All samples from the same individual yielded the same result. All amplicons were sequenced to discriminate the haplotypes. Sequencing of these amplicons revealed the occurrence of two different haplotypes (named as Ls-A and Ls-B) whose genetic identity was 99.2%. The haplotypes Ls-A and Ls-B were detected in 17.9% (14/78) and 11.5% (9/78) of the lizards sampled, respectively. We never found both haplotypes infecting the same individual as indicated by the lack of double peaks in the chromatograms from sequences obtained (see discussion). The BLAST analysis indicated that both haplotypes are close to *Eimeria arnyi* (see Fig. 2). In addition, we detected in blood samples of *Podarcis hispanica* a haplotype showing a high similarity with those isolated from *L.schreiberi* and identical to that recently reported in Portugal for the same host species (Harris et al., 2012). Specifically we amplified a DNA fragment of 1544 pb (haplotype Ph-B4 with Gen Bank accession number: JX984676) while Harris et al. (2012) report a 621 pb fragment (haplotype

667PhPO with Gen Bank accession number: JQ762306). This latter isolate corresponds to the fragment comprised between the position 256 and the position 876 in our isolate Ph-B4.

In relation with haplotype Ls-A, only 4 sporozoites were found infecting leucocytes (2 infecting lymphocytes, 1 infecting a monocyte, and 1 infecting an azurophyle. Fig. 1, E-F). However, haplotype Ls-B appears infecting white blood cells more frequently than haplotype Ls-A (haplotype A: 5.9%, N=68; haplotype B: 48.9%, N=47; Difference between two proportions $p<0,001$). Exceptionally, and always in samples containing the haplotype Ls-B, we detect azurophyles infected with two parasites. In these cases, the cytoplasm of the parasites appears more stained than in single infections in leucocytes (Fig. 1, N-O). No relationship has been found between the shape of blood stages and particular haplotype.

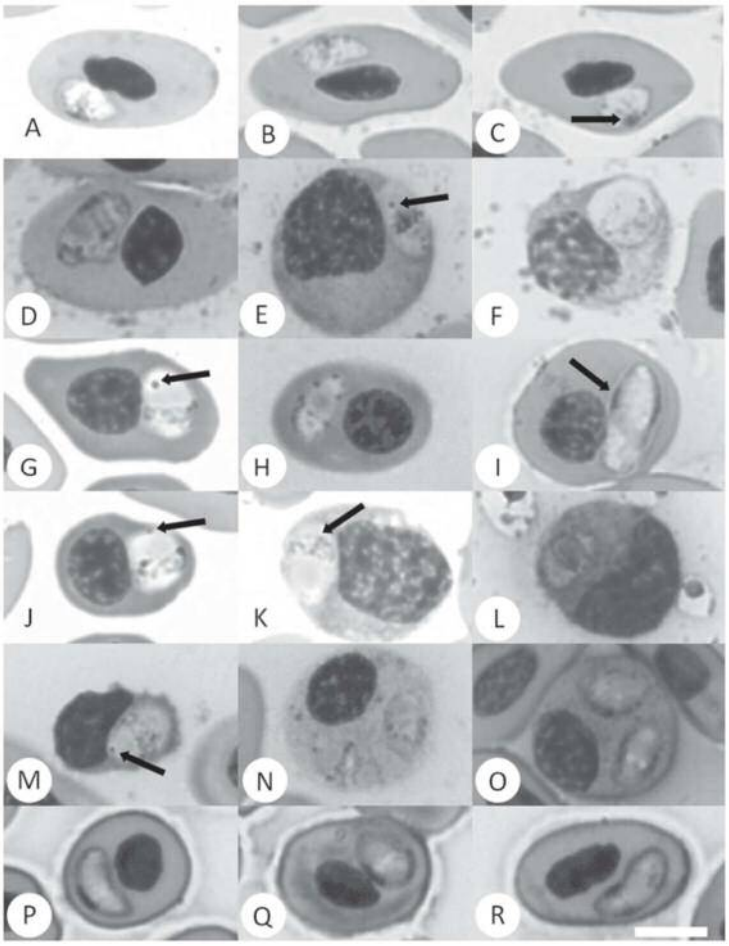

Figure 1. *Schellackia* sporozoites infecting blood cells of *Lacerta schreiberi* and *Podarcis hispanica*. Haplotype Ls-A of *L. schreiberi* (A-F). Infecting both erythrocytes (A-C) and leukocytes (D-F). Haplotype Ls-B of *L. schreiberi* (G-O). Infecting erythrocytes (G-J) and leukocytes (K-O)**.** Haplotype Ph-B4 infecting erythrocytes in *P. hispanica* (P-R). Black arrows in E, G, J and M indicate single refractile body; in C and I indicate bands of chromatine along the side of the cell; in K shows a band-like nucleus. All the pictures are shown at the same scale. Scale bar=5 µm.

Table 1. Average length and width of *Schellackia sp.* parasites of different haplotypes infecting different types of blood cells in *Lacerta schreiberi* and *Podarcis hispanica.*

	Erythrocytes				Leukocytes			
	Mean	S.D.	Range	*n*	Mean	S.D.	Range	*n*
Haplotype Ls-A								
Length	5·31	0·63	6·83–3·95	64	6·89	0·72	7·63–5·84	4
Width	3·41	0·52	4·77–2·33		4·62	0·86	5·83–3·71	
Haplotype Ls-B								
Length	5·61	0·77	8·45–4·66	24	5·77	0·72	8·14–4·82	23
Width	3·30	0·58	4·38–2·19		3·66	0·63	4·92–2·57	
Haplotype Ph-B4								
Length	5·94	0·73	5·10–6·77	5	–	–	–	–
Width	2·98	0·35	2·56–3·32		–	–	–	–

Statistical analyses comparing the length and width of parasites corresponding with haplotypes Ls-A and Ls-B do not show significant differences (one way ANOVA; length: $F_{1, 110}$= 3.787, p=0.054; and width: $F_{1, 110}$= 0.004, p=0.947). When comparing parasites infecting erythrocytes and leucocytes from both haplotypes, significant differences emerge (one way ANOVA; length: $F_{3, 108}$= 8.087, p<0.001; and width: $F_{3, 108}$= 6.869, p<0.001). A posteriori Tukey tests show that parasites from haplotype Ls-A infecting leucocytes are longer and wider than parasites from both haplotypes when infecting erythrocytes and from haplotype Ls-B infecting leucocytes. In addition, parasites corresponding to haplotype Ls-A infecting erythrocytes are shorter than parasites from haplotype Ls-B infecting leucocytes (P<0.05 in all cases).

Genetic analysis of fecal samples

The nineteen fecal samples from 2011 were analyzed by means of PCR. Six amplicons were obtained and sequenced. The BLAST analysis showed that four of them were related with the genus *Eimeria* (Genbank accession numbers: KC574076, KC574077, KC574078, KC574079) and the phylogenetic analysis grouped them within the major clade of the Eimeriidae. The other two amplicons corresponded to the genera *Adelina* (Genbank accession number: KC574080) and to an unidentified Apicomplexa (Genbank accession number: KC574081). In 2012, we found sporulated oocysts of coccidians in samples from seven individuals (7/37) by microscopical examination of fecal samples (see methods). Four of these cases correspond to *Adelina* oocysts and the DNA sequences from the other three revealed that they belong to *Eimeria* species but related to species isolated from mammals (thus probably pseudoparasites). In other words, the haplotypes obtained from these fecal parasitic stages do not correspond with those obtained from blood parasites.

Phylogenetic analysis

The Bayesian and maximum-likelihood inferences showed that the species of *Schellackia* detected in the present study form a well-supported group on its own, closely related to *Eimeria*

arnyi and *E. ranae* (Genbank sequence for *E. arnyi* was obtained from oocysts isolated from feces of the prairie ringneck snake *Diadophis punctatus arnyi* (Colubridae), Genbank accession number: AY613853, while Genbank sequence for *E. ranae* was obtained from oocysts isolated from feces of the European common frog *Rana temporaria* (Ranidae); Genbank accession number: EU717219; see Fig. 2). In addition, the haplotype detected in *Podarcis hispanica* showed a close relation with the haplotype Ls-B from *L. schreiberi*. The topology of the phylogenetic tree indicates that the group including *Schellackia* and the clade grouping *Lankesterella* and *Caryospora* are well supported but they do not share a recent common ancestor. As shown in Figure 2, *Lankesterella*, but not *Schellackia*, clusters with the clade containing majority of Stieda body-bearing eimeriids infecting birds and mammals, which is sister to the *Lankesterella-Caryospora* clade (81%).

Discussion

In the present study we genetically characterized *Schellackia* species for the first time. The phylogenetic analysis shows that they form a monophyletic cluster together with species of *Eimeria* isolated from a snake *(Diadophis punctatus arnyi)* and an amphibian *(Rana temporaria)*. The clade where the genus *Schellackia* is placed and the clade containing species of *Lankesterella* are both robustly supported and clearly separated. However, they do not share a recent common ancestor. This fact reveals that the lack of the exogenous oocyst is a characteristic that emerged independently in these two hemococcidian genera, *Lankesterella* and *Schellackia*.

Parasites detected in *Lacerta schreiberi* were identified as *Schellackia* species based on several factors. These factors are morphological characteristics (see Telford, 2008) but also the different types of cells infected, which include mainly erythrocytes but also leucocytes. Furthermore they were not detected in granulocytes as previously described for some species of the genus *Schellackia* (Telford 1993; Telford, 2008). The type of host is also an important feature as *Schellackia* is recognized as a specific parasite of lizards. However, the fact that these parasites appear closely related to *Eimeria* species isolated from *D. punctatus arnyi* and *R. temporaria* (see Fig. 2), may mean that *Schellackia* parasites observed in the smears from 2011 correspond to blood stages of a novel *Eimeria* species infecting blood of *L. schreiberi*. The *Eimeria* species of *D. punctatus arnyi* and *R. temporaria* were genetically characterized from the oocyst stages expelled with feces (Upton and Oppert, 1991; Jirku et al., 2009), a typical phase in *Eimeria* species but absent in *Schellackia* species. Thus in 2012, we reanalyzed both blood and fecal samples collected from *L. schreiberi.* However, and as expected for a *Schellackia* species, none of the fecal oocysts detected corresponded to the DNA fragment obtained from blood parasites. In every case where we detect coccidian oocysts by flotation techniques we also were able to amplify their DNA. This fact implies that the PCR performed in fecal samples was sensitive

enough to detect coccidian parasites. Therefore we can be relatively confident on the absence of exogenous oocysts corresponding with parasites detected in blood as expected for a *Schellackia* species.

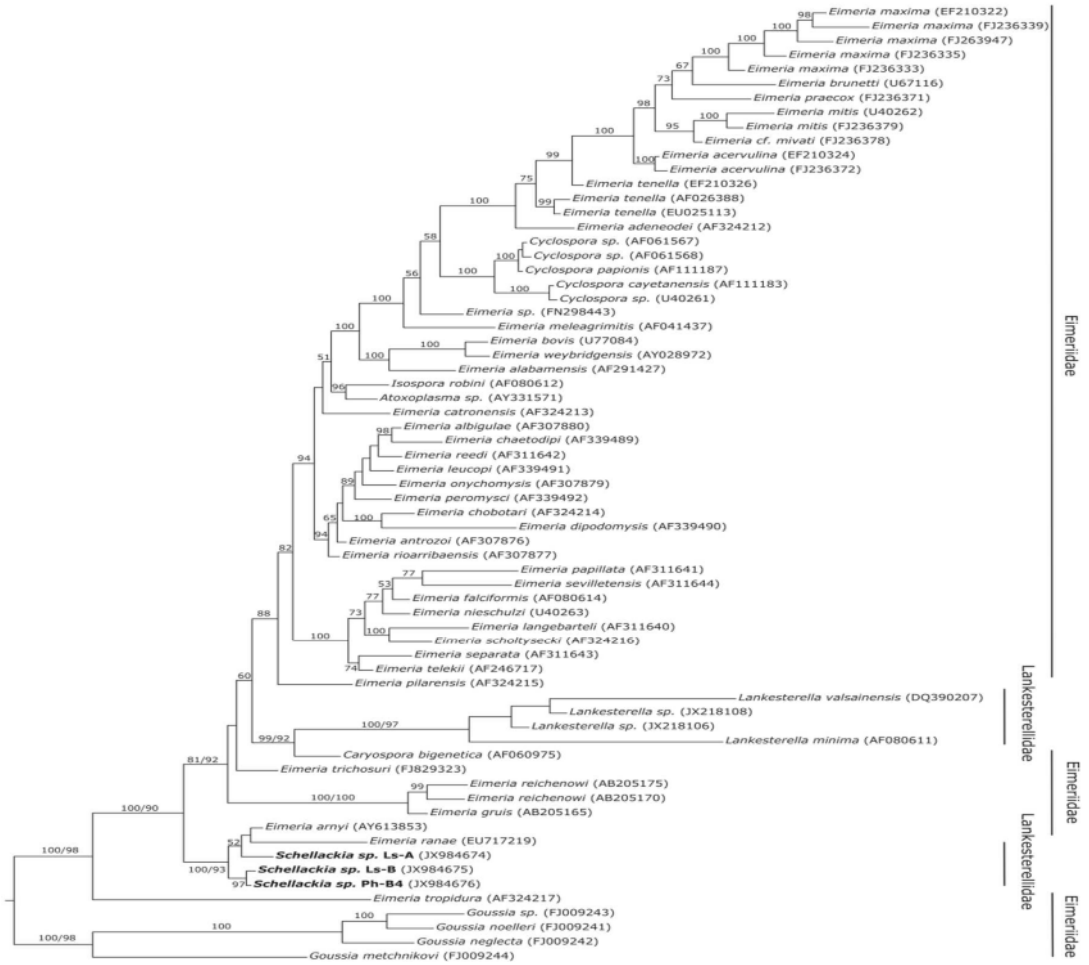

Figure 2. Bayesian inference using the GTR+G+I substitution model. This analysis consisted of 2 runs of four chains each, with 6000000 generations per run and a burn-in of 600000 generations (108000 trees for consensus tree). All branches were maintained but support values less than 50% were suppressed. All support values are percentages. Where two numbers are shown in the branch, the first one indicates the supporting value achieved by Bayesian inference and the second one by maximum-likelihood inferences (ML). The ML inference was performed using PhyML program selecting the GTR+I+G substitution model. The approximate likelihood-ratio test (aLRT) was used to obtain the clade support. The length of the alignment was 1626 bp (1208 conserved residues, 418 variables and 188 singletons). Parasite families are shown on the right and follow Upton (2000).

The genetic characterization of the sporozoites found in *L.schreiberi* showed two haplotypes whose genetic identity was 99.2%. Both haplotypes were mainly found in erythrocytes although in a few hosts, parasites were detected in both erythrocytes and leucocytes. Regardless of the type of cell parasitized, we never detected both haplotypes in the same host. However, this

fact should be taken cautiously because the sequences do not come from cloned amplicons. The lack of double peaks in the chromatograms at least indicates that if another haplotype was present in the host its intensity was very low. We found statistically significant differences in size among parasites from different haplotypes and/or infecting different host cell types. These results should be taken cautiously for parasites infecting white cells, because sample size is as low as four parasites for haplotype Ls-A. The different haplotypes detected might correspond to different developmental stages of the same parasite (see Telford, 2008), but we observed multiple stages in the same host. In addition, one haplotype (Ls-B) clusters more closely to the *Podarcis* parasite than the other (Ls-A). So it is unlikely that haplotypes Ls-A and B come from the same species. It is surprising that both haplotypes never appear coinfecting the same host, although this may be indicative of a competitive exclusion between both parasites. Our parasite sequence from *Podarcis* is identical to a previous one that was labeled as *Eimeria* (see Harris et al. 2012), which emphasizes the importance of obtaining both molecular and morphological data when identifying a parasite. The absence of a visible refractile body in all of the sporozoites of the haplotype PhB4, the pyrimorfic shape presented by these sporozoites, and the bluish stain reaction of the cytoplasm of parasites found in the slide from *P. hispanica* are traits that could be indicative of parasites recently reaching blood cells (Lainson et al., 1976). The only previous species of *Schellackia* found in the Iberian Peninsula is *Schellackia bolivari* Reichenow 1919 which is known from lacertids of different genera, i.e. *Acanthodactylus* and *Psammodromus* (Telford, 2008). However, *S. bolivari* appears to be a different species to those found in the present study because they differ with respect to the number of refractile bodies and the sort of cells infected. Molecular characterization of *S. bolivari* will help to know if they are really different species because some variation in morphometrics within the same protozoan species infecting different host could occur (see for example Merino et al. 2012).

Our results, based on the *18S* rRNA gene sequences, indicate that *Schellackia* species form a monophyletic group together with *E. ranae* and *E. arnyi*. This fact may indicate (i) the occurrence of hematic stages in those two species of *Eimeria* or (ii) that other unknown species of *Schellackia* have an exogenous oocyst stage. On the basis of the phylogenetic analysis, Lankesterellidae is not a monophyletic family. Thus the lack of exogenous oocysts is a characteristic arising independently for different lankesterellid parasites. Molecular characterization of *S. bolivari*, the type species of the genus, and the study of its phylogenetic position will allow to definitely elucidate phylogenetic affinity of this parasite genus.

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MOLECULAR CHARACTERIZATION OF HEMOCOCCIDIA GENUS *SCHELLACKIA* (APICOMPLEXA) REVEALS THE POLYPHYLETIC ORIGIN OF THE FAMILY LANKESTERELLIDAE

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Polyphyletic origin of the family Lankesterellidae

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Abstract

The current taxonomy on the hemococcidia establishes that the two genera of protozoan parasites that integrate the family Lankesterellidae are *Lankesterella* and *Schellackia*. However, the phylogeny of these genera, as well as the other coccidia, remains unresolved. In this sense, the use of type and described species is essential for the resolution of systematic conflicts. In the present study we molecularly characterize the type species of the genus *Schellackia*, i.e. *S. bolivari* from Europe and also, a described species of the same genus from Asia. At the same time, we contribute with the molecular characterization of another species of the genus *Lankesterella*. All this put together, supports the polyphyly of the family Lankesterellidae. Therefore, we propose the resurrection of the zoological family, Schellackiidae Grassé, 1953 to include species within the genus *Schellackia*.

Keywords: *Acanthodactylus;* hemococcidia; *Lankesterella;* lizard; *Schellackia, Takydromus;* phylogeny; Schellackiidae

Introduction

In 1899, Labbé described the genus *Lankesterella* in a frog species. This is a genus of apicomplexan parasites that occur primarily in amphibians around the world (Upton 2000), although there are some species within the genus *Lankesterella* described in lizards from Europe (Álvarez Calvo 1975; Chiriac & Steopoe 1977), and recent molecular studies have reported lankesterellids infecting birds (Merino *et al* 2006; Biedrzycka *et al* 2013). This genus is characterized by endogenous oocysts containing 32, or more, naked sporozoites. Later on, in 1920, Reichenow described the genus *Schellackia* in the blood cells of *Acanthodactylus vulgaris (=erythrurus)* and *Psammodromus hispanicus*, both of the family Lacertidae, in a population from Madrid, Spain. After carrying out some cross-infection experiments among individuals of both species of lizards, he concluded the conspecificity of the parasite (Reichenow 1920). The main characteristic of the genus is the formation of thin-walled oocysts in the *lamina propia* each containing eight naked sporozoites (Upton 2000; Telford 2008). In 1920, Nöller coined the name of the family Lankesterellidae that include both genera, *Lankesterella* and *Schellackia*. All species of this family are heteroxenous but sexual and asexual reproduction (i.e., merogony, gamogony, and sporogony) occur in the vertebrate host's gut. The oocysts are not expelled outside, the sporozoites are released *in situ* and pass through gut to the blood stream where they penetrate into blood cells. Thereafter, the sporozoites are ingested by hematophagous invertebrate hosts (i.e. mites, dipterans, or leeches) where they became dormant stages (Upton 2000).

In 1926, Wenyon described the subfamilies Schellackinae and Lankesterellinae within the family Lankesterellidae. Some years after, Grassé (1953) reclassify these two subfamilies as two independent families, Schellackiidae and Lankesterellidae. However, Manwell (1977) discussed the systematic level of these taxa recovering the organization proposed by Wenyon (1926). In spite of these discrepancies, in recent publications (Upton 2000, Telford 2008) the genera *Lankesterella* and *Schellackia* appear as part of the family Lankesterellidae.

The taxonomic relationship among coccidian parasites is a controversial issue, including hemococcidia (Barta 2001; Jirku *et al* 2009; Ghimire 2010). Given the fact that is not possible to identify the different genera among the hemococcidia only from the blood stages (Atkinson *et al* 2008), it is necessary the use of molecular techniques to identify these parasites from blood samples as a way to avoid killing the lizard hosts. This is important because the species of lizards are endangered and/or protected by the Spanish national law (BOE 299; Ley 42/2007). The molecular characterization of the type species of the genus *Lankesterella*, *L. minima* Chaussat 1850, was published by Barta *et al* in 2001. After that, some other *18s* rRNA gene sequences from hemococcidian parasites infecting birds and amphibians have been published (Merino *et al* 2006; Gericota *et al* 2010; Biedrzycka *et al* 2013). However, molecular data of hemococcidian parasites in reptiles are scarcely reported (Megía-Palma *et al* 2013). In the later study, the molecular

characterization of *Schellackia*-like parasites indicated that Lankesterellidae is not a monophyletic family. In this sense, the genetic characterization of the type species of this genus is essential to solve the molecular phylogeny of this group. Therefore, in the present study, we have engaged the molecular characterization of (i) the type species of the genus *Schellackia*, *S. bolivari* Reichenow 1920 isolated from one of the type host species, *Acanthodactylus erythrurus* and (ii) the described species *S. orientalis* Telford 1993 isolated from the Asian lizards of the genus *Takydromus* (Telford 1993). Additionally, we present data on a new hemoccocidian species closely related with the genus *Lankesterella* isolated in the same population of *A. erythrurus* where *S. bolivari* was found.

Material and methods

Sampling methods

In 2012, we got thirteen blood samples from a group of *Takydromus sexlineatus* individuals from a pet store that were recently imported from a farm in Indonesia. This is a host species for *Schellackia orientalis* Telford 1993 (Telford 2008). In the case of *T. sexlineatus*, we extracted the blood samples from the post orbital sinus with a heparinized microcapillar (Drummond Capillary Hematocrit 32 x 0.8 mm) in order to avoid tail loss, which is quite fragile in this lizard species. After the manipulation, all the animals stopped bleeding quickly and behaved normally. Two samples were obtained from each lizard: blood smears were made from one drop of the sample, while the remaining blood was preserved in Whatman FTA Classic Cards (FTA® Classic Card, Cat. No. WB12 0205). The FTA cards were stored in plastic bags with silica gel for later DNA extraction. All blood smears were immediately air dried and later, within the same day, fixed with absolute methanol (Svahn 1975). All blood smears were stained with Giemsa stain (1/10 v/v) for 45 minutes. Slides were examined for hemoparasites following Merino & Potti (1995).

During the field season of 2013, we captured 10 individual lizards of *Acanthodactylus erythrurus*, in a bushy area in Madrid (39° 59' 40.362", -3° 37' 17.1804"). We chose the sampling area, close to the city of Madrid, following the original description of the type species, *S. bolivari* (Reichenow 1920). Blood samples were taken from the ventral vein at the base of the tail (Salkeld and Schwarzkopf 2005) by puncture, using a syringe needle (BD Microlance 3; 23G: 0.6 x 25 mm) and picking up the blood with a capillary tube (BRAND, Micro-Haematocrit Tubes, 75 x 1.1 mm, Na-Heparinized). The skin around the area of puncture was previously cleaned with ethanol 96%, to avoid potential fecal contamination. Blood samples were preserved as described above for *T. sexlineatus*. All the *Acanthodactylus* lizards were released after manipulation in the original sampling site.

Molecular methods

We extracted genomic DNA from blood preserved on FTA cards following the protocol described in Megía-Palma *et al* (2013). Thereafter, the DNA was purified using the UltraClean GelSpin DNA Purification kit (MO BIO). The PCR settings and primers used to perform the molecular screening to detect *Schellackia* are detailed in supporting information on-line (see also Megía-Palma *et al* 2103). All amplicons were sequenced to discriminate the haplotypes.

The three DNA sequences (*18S* rRNA) obtained from the lizards were aligned together with other 68 sequences included in a previous study (Megia-Palma *et al* 2013). The alignment was performed using PROBCONS (http://toolkit.tuebingen.mpg.de/probcons). Poorly aligned positions and divergent regions of the alignment were suppressed using GBlocks program (Talavera and Castresana 2007) selecting the following options: "Minimum Number of Sequences for a Conserved Position" to 36, "Minimum Number of Sequences for a Flank Position" to 36, "Maximum Number of Contiguous Nonconserved Positions" to eight, "Minimum Length of a Block" to 10, and "Allowed Gap Positions" to "With Half". The final alignment contained 1477 positions and 71 sequences. The substitution model GTR+I+G was selected to perform the Bayesian analysis. This analysis consisted of two runs of four chains each, with 10,000,000 generations per run and a burn-in of 2,500,000 generations (150,000 trees for consensus tree). The final standard deviation of the split frequencies was 0.01 in both analyses. Convergence was checked using the Tracer v1.5 software (Rambaut & Drummond 2007). All of the model parameters were higher than 100.

To evaluate the relationships of *S. bolivari* to its sister taxa in more detail, a file containing only 16 sequences was analyzed. The alignment and Bayesian analysis were performed as commented above. The final alignment contained 1,563 positions. In this case, the phylogenetic analysis consisted of two runs of four chains each, with just 1,000,000 generations per run and a burn-in of 250,000 generations (15,000 trees for consensus tree).

In addition, both alignments were analyzed using the maximum-likelihood inference (PhyML program; Guindon *et al* 2010). This analysis was performed with the two alignments. The substitution models were those indicated above, the subtree pruning and regrafting (SPR) and the nearest-neighbor interchange (NNI) tree-rearrangements were selected, and a Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support (Anisimova et al. 2011).

Microscopic methods

The intensity of infection in the blood smears was calculated counting the total number of cells infected per 10.000 erythrocytes (Stuart-Fox *et al* 2010). In order to estimate differences in size between the sporozoites of *S. bolivari* and the lankesterellid, several morphometric measurements were taken from pictures obtained from the parasites found in slides where the molecular methods

had shown simple infections. Pictures of parasites were taken with an adjustable camera for microscope (Olympus SC30) incorporated to a microscope U-CMAD3 (Olympus, Japan). The length and the width of the intracellular parasites, as well as the length of the nucleus and the refractile bodies, were measured with the MB-ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/).

Results

We observed sporozoites infecting erythrocytes in five of the 10 (5/10) thin blood smears of *Acanthodactylus erythrurus*. The mean intensity per 10,000 erythrocytes in the five positive smears was 27.8. The higher intensity was 115/10,000 erythrocytes, and the lower 1/10,000. The sequences obtained from the five infected individuals revealed the occurrence of three haplotypes named Ae-M, Ae-S and Ae-Lk (Genbank accession numbers: Ae-M: KJ131415; Ae-S: KJ131416 and Ae-Lk: KJ131417). Two of them differing in just four bases (Ae-M and Ae-S; identity 99.7%) and the third (Ae-Lk) presented a genetic identity of 96.3% and 96.1% with Ae-M and Ae-S haplotypes, respectively. On the one hand, the phylogenetic analysis clustered the haplotypes Ae-M and Ae-S together with *Schellackia*-like parasites indicating that they belong to *S. bolivari* (see Fig. 1). As can be seen in the same figure, the genus *Schellackia* has not a monophyletic origin due to the occurrence of *Eimeria arnyi* and *E. ranae* in the same clade. The analysis restricted to 16 different sequences, in order to solve phylogenetically this group, showed *E. ranae* as a sister group of the genus *Schellackia*. However *E. arnyi* shared a common ancestor with the genus (Fig. 2). On the other hand, the haplotype Ae-Lk groups with a strong support with the available sequences of the genus *Lankesterella* (Fig. 1).

Before conducting the morphological description of the parasites, the infected individuals were analyzed using specific primers (see supporting information on-line), we detected one individual exclusively parasitized by the haplotypes Ae-M and Ae-S (i.e., *S. bolivari*), other two by haplotype Ae-Lk (i.e., lankesterellid), and other two presented a mixed infection.

There were two clearly different parasite morphologies in the blood smears where simple infections were confirmed by molecular methods. The parasitic stages corresponding to *Schellackia* showed an elongated pyriform shape. Commonly, a pointed end is present, where the single refractile body of the sporozoite is located. It presents a characteristic bluish stain. On the opposite side, the end is rounded. The nucleus is diffuse, as in other species of *Schellackia* previously described (Telford 2008) (see Fig. 3). The presence of the sporozoite within the cytoplasm of the erythrocyte does not seem to distort the cytoplasmatic wall of the host cell. Furthermore, these sporozoites do not displace the nucleus of the host cell as much as it happens in some other infections by hemoparasites (e.g. *Hepatozoon* spp.) (see Telford 2008). We deposited voucher blood smears with simple infection of *S. bolivari* and *Lankesterella* sp. in the

invertebrate collection of the Museo Nacional de Ciencias Naturales-CSIC in Madrid *(Lankesterella* sp. MNCN 35.63; *S. bolivari* MNCN 35.62).

In the corresponding blood smears where the PCR had revealed a simple infection by the lankesterellid, the morphology of the sporozoites is further different from those where a simple infection by *Schellackia* was found (see Table 2). The common shape presented by these parasites goes from somewhat triangular to elongate. The length is always longer than the sporozoites of *Schellackia* sp. ($F(1, 202)=220.74$; $p<0.00001$). The nucleus appears like disperse granules of cromatine in the middle of two prominent refractile bodies which stain pale blue as compared to the cromatine. In 54.6% of the sporozoites (N=119), there are azurophilic granules throughout the cytoplasm of the protozoa and along the surface of the refractile bodies (see Fig. 4).

Figure 1. Bayesian inference using the GTR+G+I substitution model. This analysis consisted of 2 runs of 4 chains each, with 10000000 generations per run and a burn-in of 2500000 generations (150000 trees for consensus tree). All branches were maintained but support values less than 50% were suppressed. Where two numbers are shown in the branch, the first one indicates the supporting value achieved by Bayesian inference and the second one by maximum-likelihood inferences (ML). The ML inference was performed using PhyML program selecting the GTR+I+G substitution model. Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support. The length of the alignment was 1477 bp. Asterisk in E. ranae and E. arnyi indicates the species which misidentification might be probably due to the presence of haemococcidia in the sample (see Discussion).

In the case of *Takydromus sexlineatus*, we observed sporozoites of *Schellackia orientalis* in three of the thirteen lizards sampled. In one of the three individual lizards, the infection occurred in both erythrocytes and leukocytes (see Fig. 5). In the case of the erythrocytes, single infections were always observed. While in the leukocytes we observed multiple infections until a number of six sporozoites. A single refractile body is present and the sporozoites, infecting leukocytes, are surrounded by a parasitophorus vacuole (Fig. 5, E-O).

Discussion

The hemococcidians gather two genera of apicomplexan protozoa whose sporozoite morphologies are indistinguishable (Atkinson *et al* 2008). However, in the present study we found two different morphotypes of hemococcidians infecting *Acanthodactylus erythrurus* from Spain. One of them presented larger sporozoites and two obvious refractile bodies while the other were shorter in length and the unique refractile body was near to the apical part of the sporozoite. When Reichenow (1920) described for the first time *Schellackia bolivari* as the type species of the genus, he highlighted the fact that the sporozoites showed two refractile bodies (see Reichenow 1920). However, the molecular analysis of the samples from individuals parasitized with a single infection exhibiting sporozoites with two clear refractile bodies, as in the original description, revealed that this morphotype corresponds to a new species closely related to the genus *Lankesterella*. As it forms a highly supported monophyletic clade together with *Lankesterella* species, probably this morphotype corresponds with the first *Lankesterella* species isolated from lizards. On the other hand, the sporozoites with just one refractile body genetically correspond to the genus *Schellackia*, and therefore, the morphological description of these sporozoites corresponds to *S. bolivari*. We assigned two haplotypes, Ae-M and Ae-S, differing only in 4 bases to *S. bolivari*. Other studies have found Apicomplexa parasites yielding different *18S* rRNA products in the same host (Li *et al* 1997) and the same process has been suggested to explain the genetic variability found within some hemogregarines (Perkins & Keller 2001; Starkey *et al* 2013).

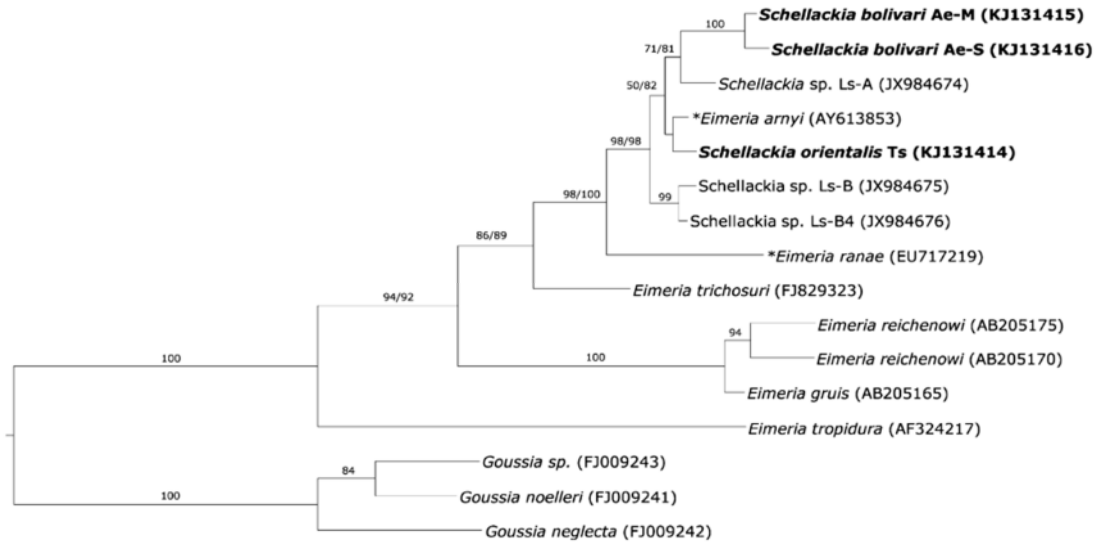

Figure 2. Evolutionary relationships between *S. bolivari* and its sister taxa. Bayesian inference using the GTR+G+I substitution model. This analysis consisted of 2 runs of 4 chains each, with 1000000 generations per run and a burn-in of 250000 generations (15000 trees for consensus tree). All branches were maintained but support values less than 50% were suppressed. Where two numbers are shown in the branch, the first one indicates the supporting value achieved by Bayesian inference and the second one by maximum-likelihood inferences (ML). The ML inference was performed using PhyML program selecting the GTR+I+G substitution model. Bayesian-like transformation of aLRT (aBayes)was used to obtain the clade support. The length of the alignment was 1563 bp. Asterisk in *E. ranae* and *E. arnyi* indicates the species which missidentification may be probably due to the presence of haemococcidia in the simple (see Discussion).

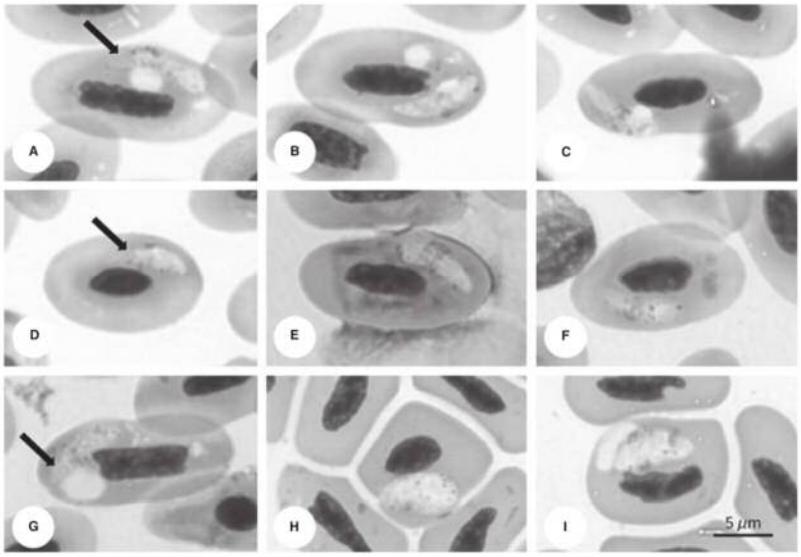

Figure 3. *Schellackia bolivari* sporozoites infecting erythrocytes in *Acanthodactylus erythrurus* from Madrid. Black arrows in A, D and G indicates the single refractile body in the anterior part of the sporozoite. All the pictures are shown at the same scale.

Table 1. Morphological data of the sporozoites (S) and refractile bodies (RB) of the haemococcidia detected in *Acanthodactylus erythrurus*. See Telford (1993) for the original description of *S. orientalis.* No related data is reported in Reichenow (1920) for *S.bolivari*. *Schellackia bolivari* and *S. orientalis* show only one refractile body per parasite while the *Lankesterella* species shows two.

	Schellackia bolivari				*Lankesterella* sp.				*Schellackia orientalis* in erythrocytes				*Schellackia orientalis* in leucocytes			
	N	Mean	SD	Range	N	Mean	SD	Range	N	Mean	SD	Range	N	Mean	SD	Range
S width (µm)	105	3.05	0.84	1.45–5.58	129	3.14	0.67	1.78–4.92	7	2.55	0.63	1.88–3.5	32	3.58	0.52	2.7–4.77
S length (µm)	105	7.37	0.94	5.13–10.9	129	9.46	0.93	6.71–11.81	7	5.9	0.59	5.05–6.52	32	7.11	0.89	4.97–9.04
RB diameter (µm)	*	*	*	*	129	1.6	0.25	0.87–2.27	6	1.61	0.37	1.12–2.35	26	2.4	0.42	1.55–3.62

*The diffuse outline of the refractile bodies makes difficult to obtain accurate measures.

Taken together, the original description of *S. bolivari* was probably performed from individuals with mixed infection insomuch as Reichenow (1920) reported the presence of endogenous oocysts containing eight nuclei, stage that defines the genus *Schellackia* (Upton 2000; Telford 2008). Unfortunately, we cannot compare the size of the sporozoites found in our blood samples (see Table 2) with those found in the original description, since (i) Reichenow did not report useful data on this sense and (ii) the holotype of the original description seems to be lost. Only a general sporozoite length size (5.2 µm) was provided (Reichenow 1920 in Telford 2008), but no standard deviation or number of measured sporozoites was given which prevents statistical analysis.

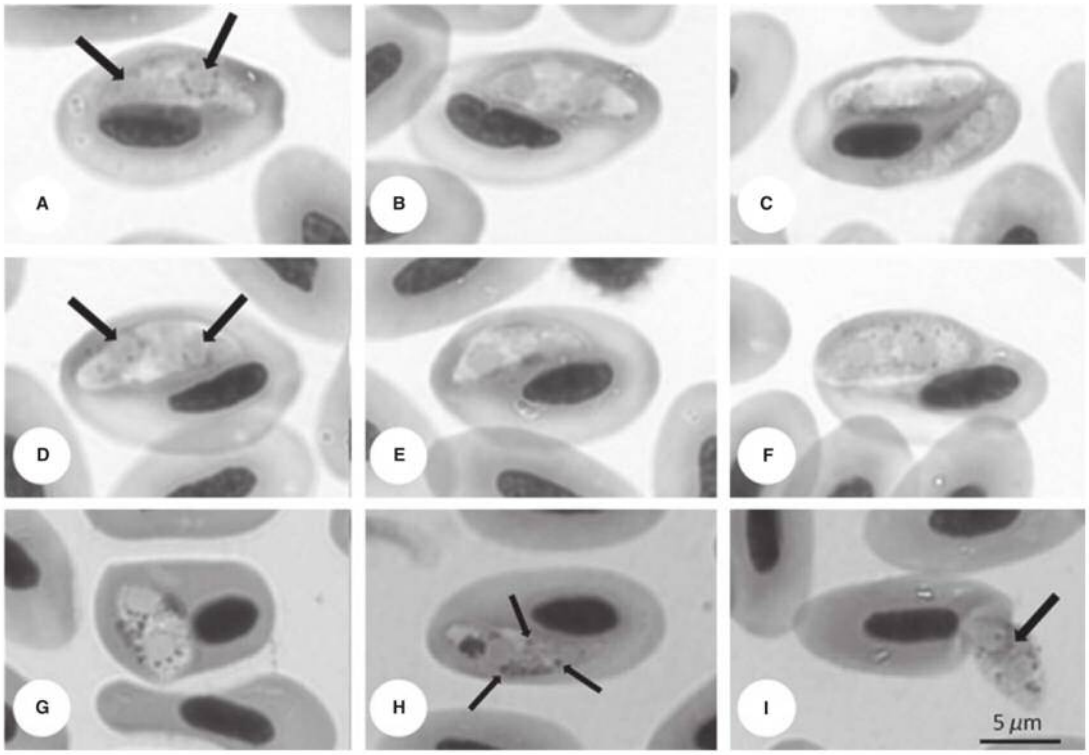

Figure 4. *Lankesterella* sp. sporozoites infecting erythrocytes in *A. erythrurus* from Madrid. Black arrows in A and D indicates the two refractile bodies to both sides of the nucleus of the sporozoite. In I, the black arrow indicates the nucleus. The three small black arrows in H indicate the granules of chromatine that can be seen in several pictures (A, B, D, F, G, H and I). All the pictures are shown at the same scale.

In relation with the taxonomy of the genus *Schellackia*, at the present time there are nine described species distributed worldwide which exhibit a variable number of refractile bodies in the cytoplasm of the sporozoites. For example, *S. brygooi*, *S. orientalis*, *S. occidentalis* and *S. golvani* show one refractile body, while *S. agamae* and *S. ptyodactyli* show two of them (Telford 2008). In the case of *S. landaue* and *S. calotesi* the number of refractile bodies goes up till two (Telford 2008). Considering the number of refractile bodies present in these species, and the case study presented in this work, it may be useful to accomplish the molecular characterization of these species, to clarify the taxonomy of the group. This molecular study on the current known species within the lankesterellids would also help to i) clarify whether the original description of these species would have been performed from individual hosts parasitized by mixed infections or not, and ii) whether the number of refractile bodies in the sporozoites within the species of the family Lankesterellidae may be an useful trait to diagnose the genera *Schellackia* and *Lankesterella*.

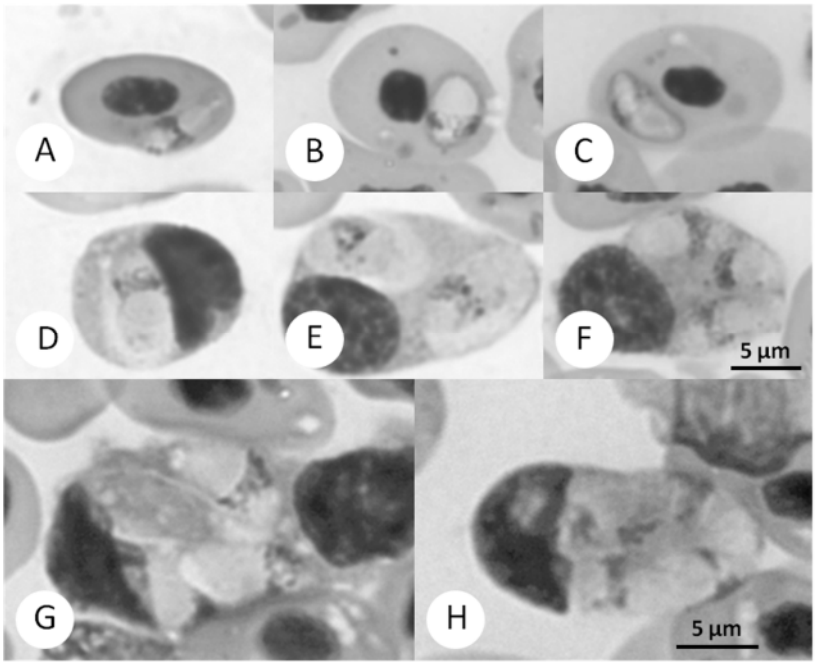

Figure 5. *Schellackia orientalis* sporozoites infecting both erythrocytes (A-C) and leukocytes (D-H) in *Takydromus sexlineatus.* In leukocytes commonly multiple infections can be seen (E-H). A-F and G-H are made at the same scale.

The phylogenetic analysis based on the *18s* rRNA gene sequences shows that *S. bolivari* and *S. orientalis* cluster with other *Schellackia*-like parasites previously isolated from lizards of the genera *Lacerta* and *Podarcis*. This group is clearly separated from that containing the genus *Lankesterella*, confirming the polyphyletic origin of the family Lankesterellidae as suggested in a previous work (see Megia-Palma *et al* 2013). However, the monophyletic origin of the genus *Schellackia* is not supported either due to the occurrence of *Eimeria arnyi* and *E. ranae* in the same clade (see Fig. 2). The presence of these two species of *Eimeria* in this clade, grouped along with several gene sequences of *Schellackia,* suggests the misidentification of *E. ranae* and *E. arnyi* with species of the genus *Schellackia*. This possibility could be due to contamination of the samples with hemococcidian protozoa, which accomplish their life cycle in the intestinal tissues (Upton 2000). This could be the case for *E. ranae*, which was obtained from "mashed intestine of a tadpole" (Jirku *et al* 2009) and its SSU sDNA was amplified using universal eukaryotic primers (Medlin *et al* 1988 in Jirku 2009). Moreover, *Schellackia* has been described parasitizing frogs before (i.e. Paperna and Lainson 1995). The case of *E. arnyi* is surprising as it host is the prairie ringneck snake and no *Schellackia* species is known to infect ophidians. However, some hematic coccidia are able to infect predator tissues after prey swallowing (Tomé *et al* 2013), and this is a characteristic present in lankesterellids life cycles (Klein *et al* 1988, Bristovetzky and Paperna 1990). Thus the possibility of snakes being infected by lankesterellids after consumption of an infected prey exists. That being the truth, the presence of small amounts of blood cells in fecal

samples may lead to molecular misidentification of intestinal parasites (pers. obs.). If sequences of *E. ranae* and *E. arnyi*, were confirmed to belong to the genus *Schellackia*, the monophyly of this genus along with its independent origin from other lankesterellids, would justify the resurrection of the family Schellackiidae Grassé, 1953.

In conclusion, the data presented in this study have confirmed the polyphyletic origin of the family Lankesterellidae. In addition, we morphologically described the hematic stages (i.e., sporozoites) of *S. bolivari*, which allowed us to compare them with the original description of the type species. This comparison, together with the molecular analyses of infections by parasites with different morphologies, shows that the blood stages described by Reichenow (1920) belonged, in fact, to the genus *Lankesterella*. However, in the case that *E. ranae* and *E. arnyi* were confirmed to be species within the genus *Schellackia*, we suggest a revision of the status of the family Lankesterellidae, with the resurrection of the family Schellackiidae Grassé, 1953.

Supplementary information

Pairs of primers used in the present study.

Primers	Sequence 5'→ 3'	Size (bp)	Annealing	Extension (s)	Parasites (specificity)
BT-F1 [1]	GGTTGATCCTGCCAGTAGT	1050	58°C	80	*Schellackia* / Lankesterellid
EimRodR [1]	GCATTTCCCTATCTCTAGTCGG				
Hep600F1 [1]	TCGTAGTTGGATTTCTGTCG	1003	58°C	80	*Schellackia / Lankesterellid*
Hep1600R [1]	AAAGGGCAGGGACGTAATCGG				
SbolF2 [2]	CGTAGTTGGATTTCTGTCGAGG	429	58°C	40	*Schellackia* (haplotype Ae-S)
EimRodR [1]	See above				
Hep50F [1]	GAAACTGCGAATGGCTCATT	656	58°C	50	Lankesterellid
AcanR [3]	GTACCTGACAACGCAATTAAG				
BT-F1 [1]	See above	706	58°C	50	*Schellackia*
SbolR [4]	GGAAGGAACCGGAAGAATGC				

[1] Primers used by Megia-Palma et al. (2013) to detect *Schellackia*-like parasites.

[2] Primer designed in the present study to differentiate the two *Schellackia* haplotypes.

[3] Primer designed in the present study to specifically detect the lankesterellid haplotype.

[4] Primer designed in the present study to specifically detect the genus *Schellackia*.

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work. The Spanish Ministerio de Ciencia e Innovacion provided financial support for our research (project CGL2012-40026-C02-01 to S. M. and J. M. and grant number BES-2010-038427 to R. M.). All permissions for collecting specimens were obtained from the corresponding authorities.

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MOLECULAR DIVERSITY OF THE GENUS *SCHELLACKIA* (APICOMPLEXA: SCHELLACKIIDAE) PARASITIZING LIZARDS OF THE FAMILY LACERTIDAE (SQUAMATA)

Short communication

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Abstract

Parasites of the genus *Schellackia* primarily infect lizards around the world. The current number of described species is low but its geographic distribution covers all continents inhabited by lizards. However, so far only five haplotypes belonging to this genus were described. In this study, we screened 17 different lizard species in a restricted geographic area in Southwestern Europe and North Africa and found 18 haplotypes of the gene *18S* rRNA belonging to the genus *Schellackia*. *Schellackia* haplotypes exhibited a high degree of host genus specificity since no cross-infection among lizard genera was found. One striking example of host specificity has been detected in the host genus *Podarcis*. We found six *Schellackia* haplotypes in seven *Podarcis* species, along the entire sampling range covering from the Chafarinas Islands to the Pyrenees. However, none of these haplotypes was found in any other host genus despite their geographic distribution overlap with *Podarcis*. The molecular diversity of *Schellackia* parasites and the host specificity found here suggest that this genus is more diverse and host specific than previously thought.

Ten species of genus *Schellackia* (Apicomplexa: Schellackiidae) are known to infect lizards around the world (Ávarez-Calvo et al., 1975; Telford, 2008). The type species, *Schellackia bolivari* Reichenow 1920, was described parasitizing either the spiny-footed lizard *Acanthodactylus erythrurus* Schinz 1833 (Squamata: Lacertidae), and the Spanish *Psammodromus*, *Psammodromus hispanicus* Fitzinger 1826 (Squamata: Lacertidae) from the Iberian Peninsula. In addition, *S. bocagei* was lately described (Álvarez-Calvo, 1975) as parasite in the Andalusian wall lizard *Podarcis vaucheri* Boulenger 1905. The small number of characteristics that can be described from the sporozoites found in blood cells of the definitive host makes difficult to describe new species, and most of the key characters used to describe them are restricted to the endogenous stages in the gut tissue of the definitive host are used to describe them (Reichenow, 1920; Bonorris and Ball, 1955; Rogier and Landau, 1975; Bristovetzky and Paperna, 1990; Paperna and Finkelman, 1996; Telford, 1993, 2008). However, in recent studies, parasites of the genus *Schellackia* found in the Schreiber's Green lizard *Lacerta schreiberi* Bedriaga 1878, the Guadarrama wall lizard *Podarcis guadarramae* (Boscá, 1916) Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet 2014, and the spiny-footed lizard *Acanthodactylus erythrurus* from the Iberian Peninsula were molecularly characterized (Megía-Palma et al., 2013, 2014). These parasites are phylogenetically related to *S. orientalis* Telford 1993 found in *Takydromus sexlineatus* Daudin 1802 from Thailand.

In a survey in the Iberian Peninsula and the North of Africa, we obtained 919 blood samples from 17 species of lizards belonging to family Lacertidae. In addition, we sampled seven localities distributed along the entire distribution of the type host species, the spiny-footed lizard *Acanthodactylus erythrurus* including one locality in Morocco (Figure 1a). After blood sampling, all lizards were safely released in the same area where they had been captured. The methods for (i) extraction and preservation of blood samples, (ii) the microscopic study of thin blood smears of the lizards, (iii) extraction of the parasite DNA for molecular screening, and (iv) phylogenetic analyses of the parasites of the genus *Schellackia* are explained in Megía-Palma et al. (2013 and 2014).

We found 256 individuals of fifteen lacertid species infected by *Schellackia* parasites of similar morphologic characteristics. Infections by parasites of this genus were not detected in *P. carbonelli* Pérez-Mellado 1981 from Huelva (Figure 1b) or *Psammodromus hispanicus* (s.l.) from Segovia and Toledo (Figure 1c). All the blood smears that were positive for *Schellackia* parasites presented sporozoites that were morphologically compatible with those of *S. bolivari* (Reichenow, 1920; see discussion in Megía-Palma et al., 2014).

In particular, we observed single refractile bodies in the sporozoites present in the red blood cells of the fifteen lacertid species that were host for *Schellackia* parasites (Figure 2). However, the molecular characterization of the samples revealed the presence of 18 variants of the *Schellackia 18S*

rRNA gene. Four of the host genera surveyed here were infected by two or more parasite haplotypes. Specifically, *Lacerta schreiberi* in Segovia was infected by two different haplotypes, LsA and LsB (see also Megía-Palma et al., 2013).

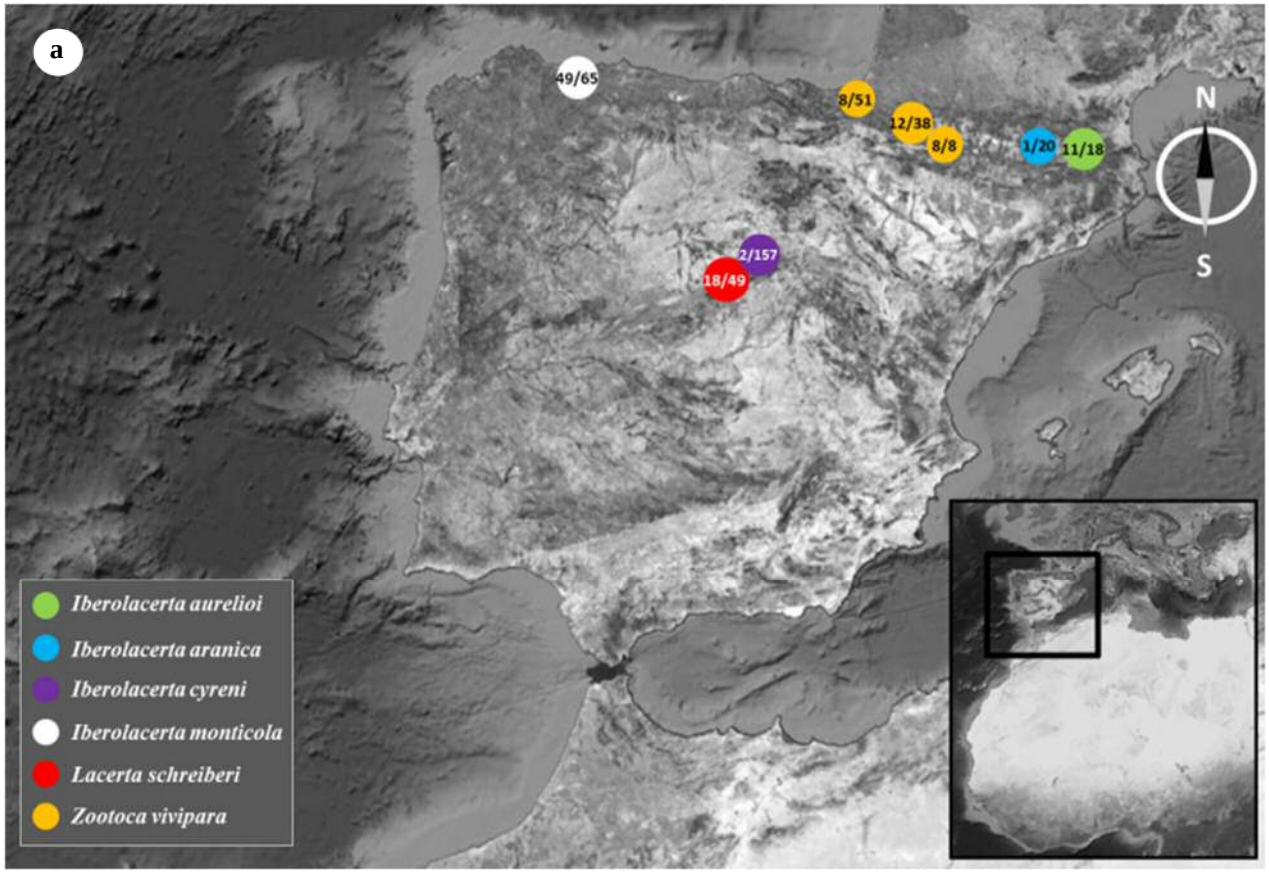

Figure 1a. Proportion of infected individuals in each population sampled. The colours represent different lacertid species. Localities for species of the genera *Iberolacerta*, *Lacerta* and *Zootoca.*

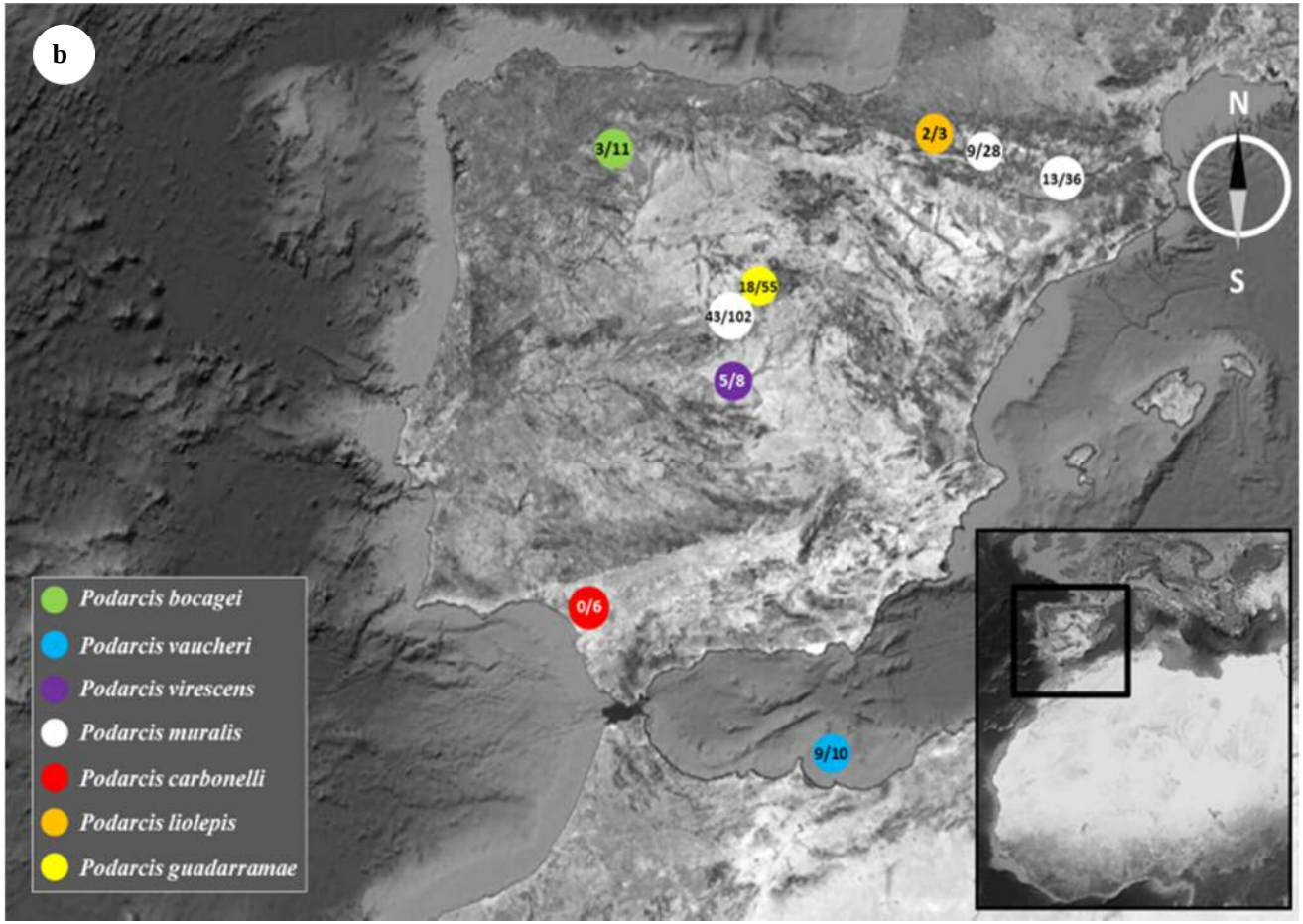

Figure 1b. Proportion of infected individuals in each population sampled. The colours represent different lacertid species. Localities for species of the genus *Podarcis*.

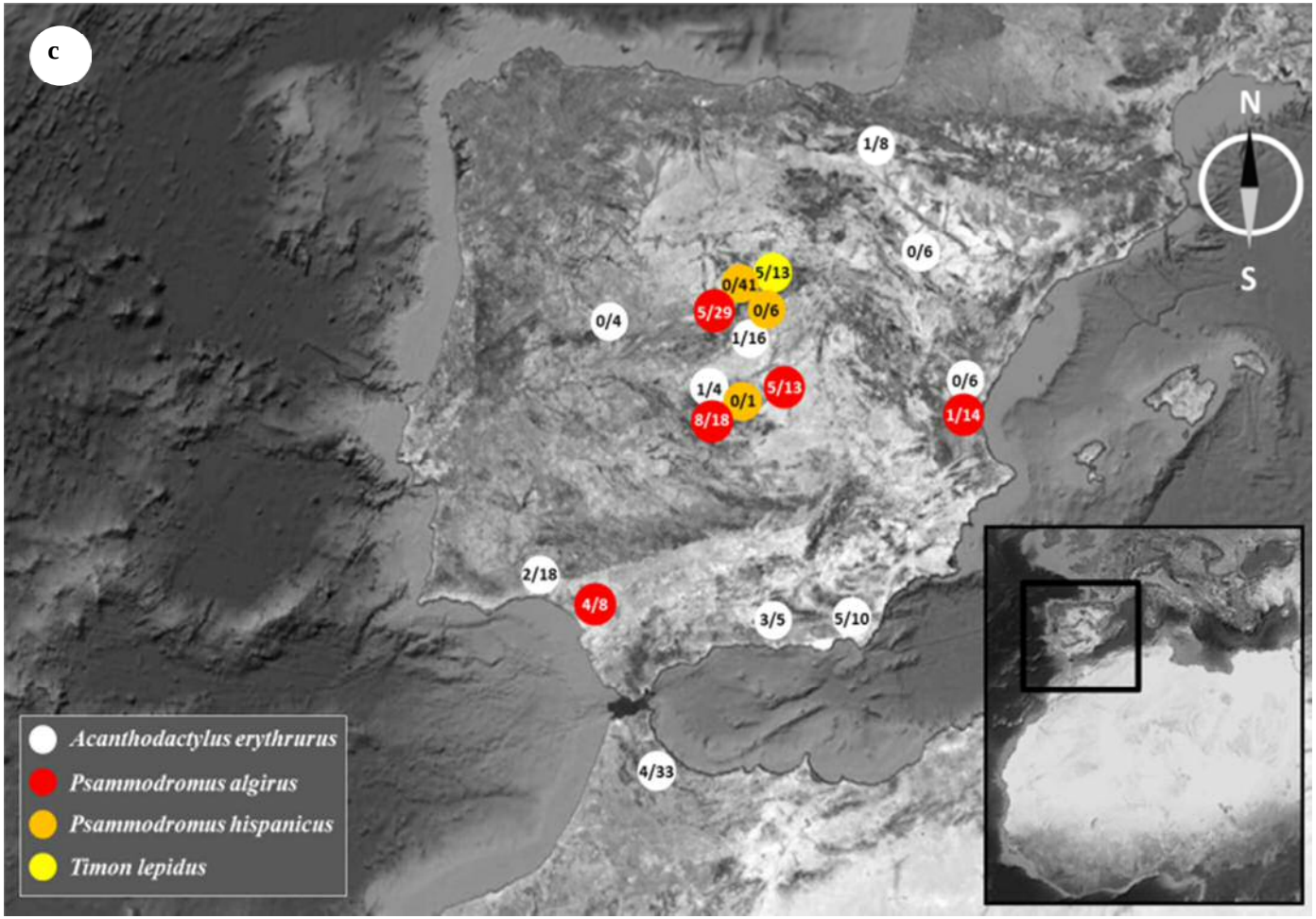

Figure 1c. Proportion of infected individuals in each population sampled. The colours represent different lacertid species. Localities for species in the genera *Acanthodactylus*, *Psammodromus* and *Timon*.

The phylogenetic relationships of these haplotypes were not resolved (Figure 3) but they infect different blood cell types (see Megía-Palma et al., 2013). These parasite haplotypes were present in the same population in Segovia, although they did not infect the same host individuals (Megía-Palma et al., 2013). Similarly, we surveyed three populations of *Zootoca vivipara* in the Pyrenees. In two of the populations (50 individuals per population, Somport and Portalet, in Huesca), we found two parasite haplotypes of *Schellackia* (Z1 and Z2) but we found only one of them (Z1) in the population from Irún, Guipúzcoa (N=50 lizards). In addition, we consistently found two haplotypes of the *18S* rRNA gene of *S. bolivari* (AeM and AeS, Megía-Palma et al., 2014) of *S. bolivari*, parasitizing blood cells in *A. erythrurus* across the sampling sites for this host species. In a similar way, we repeatedly found a single *Schellackia 18S* rRNA gene haplotype (Ps1) infecting *Psammodromus algirus* Linnaeus 1758 in several localities (i.e. Aranjuez, Sevilla, Segovia, Toledo and Valencia). The haplotypes respectively found in the spiny-footed lizard and the large *Psammodromus* species were not found in any other lizard species along the distributional range of these hosts suggesting a high host-specificity of *Schellackia* parasites. Indeed, one striking case of the specificity of *Schellackia* parasites is the host genus *Podarcis* where we found six variants of the gene *18S* rRNA of *Schellackia* parasites consistently distributed along the sampling range of this host genus that covered seven host species. Specifically, the parasite haplotype P3 was found in *P. virescens* Geniez, Sá-Sousa, Guillaume, Cluchier and Crochet 2014 from Toledo, *P. bocagei* Seoane 1885 from León, *P. vaucheri* from Chafarinas and *P. muralis* Laurenti 1768 from the Sistema Central Mountains. Whereas the *Schellackia* haplotype P1 was found in *P. liolepis* Boulenger 1905 and *P. muralis* from the Pyrenees, and *P. guadarramae* from either slopes of the Guadarrama Mountains in Madrid and Segovia. The remaining four variants of the parasitic gene were found in *P. guadarramae* from Segovia (P1a and P4), *P. virescens* from Toledo and *P. muralis* from the Pyrenees (P1b) and the Guadarrama Mountains in Madrid (P2). This molecular diversity of parasites of the *Podarcis* complex might reflect the haplotypic diversity of the host (Harris and Sá-Sousa, 2002; Pinho et al., 2004) which is considered to be rapidly radiating (Pinho et al., 2008; Geniez et al., 2014). The phylogenetic analyses (Figure 3) revealed two sister clades grouping *Schellackia* parasites found in lacertids. One of them grouped parasites found in *A. erythrurus* (*S. bolivari*), *Z. vivipara* and *T. sexlineatus* (*S. orientalis*). The other clade showed that *Schellackia* parasites found in lizard species of the genus *Podarcis* were closely related to parasites found in lizards of the genus *Iberolacerta.* More specifically, parasites found in the subgenus *Pyrenosaura* (*Iberolacerta aranica* Arribas 1993 and *I. aurelioi* Arribas 1994) from the Pyrenees (IB63) were closely related to the haplotypes P3 and P4 found in *Podarcis* from Chafarinas, Toledo, Segovia and León. Whereas the haplotypes found in *I. monticola* Mertens 1929 from Asturias and León (IB28) and *I. cyreni* Müller and Hellmich 1937 from the Guadarrama Mountains (IB244) were closely related

to the haplotypes P1, P1a, P1b and P2 found in *Podarcis* host species from the Pyrenees and Madrid.

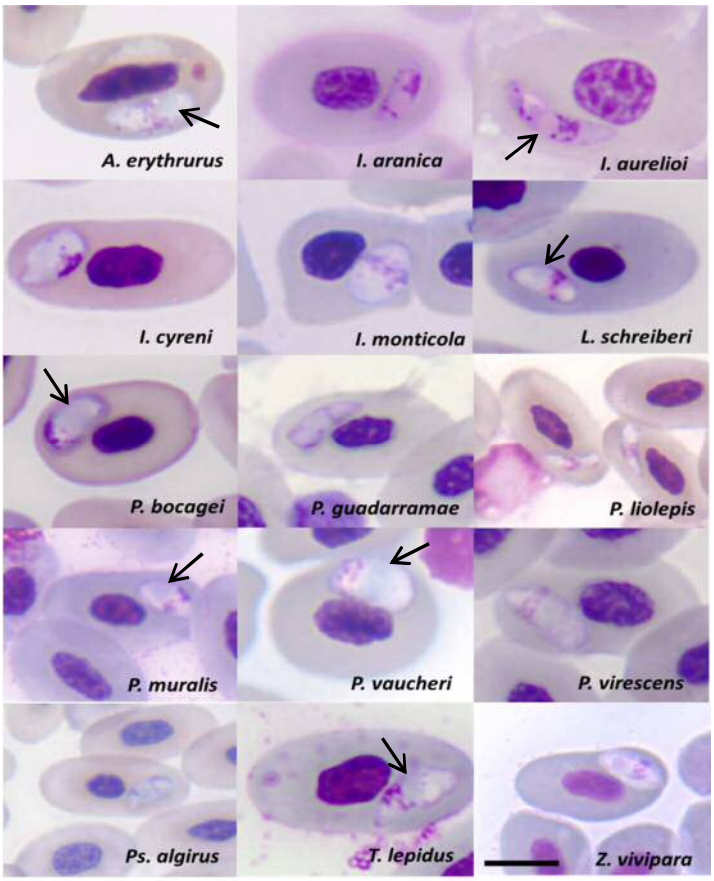

Figure 2. Microphotographs of sporozoites of the genus *Schellackia* in erythrocytes of lacertids in the Iberian Peninsula and the North of Africa. Black arrows indicate some examples of the single refractile body observed in these parasitic stages. All pictures were taken at 1000X magnification and are shown at the same scale. **Scale bar= 5 µm.**

The results of this study allow us to conclude that the diversity and specificity of the parasites of the genus *Schellackia* may be higher than it was previously thought. Some of the host species included in this study shared the same habitat and sometimes the same niche. However, the specificity of parasites of the genus *Schellackia* was high and no cross-infection was detected at the genus host level. This molecular diversity of parasites of the genus *Schellackia* might be evidencing differences in the ecological requirements of their definitive or intermediate hosts that drove processes of evolutionary radiation and may reflect co-evolutionary host-parasite relationships (e.g. Hafner and Nadler, 1998). Hence, the reproductive isolation of these parasites with ancient host-parasitic relationships may reflect the former lost in genetic flux of their hosts. Therefore, further studies on the phylogenetic relationships of these parasites and their vertebrate and invertebrate hosts may help understand the evolution of these herp-specific parasites.

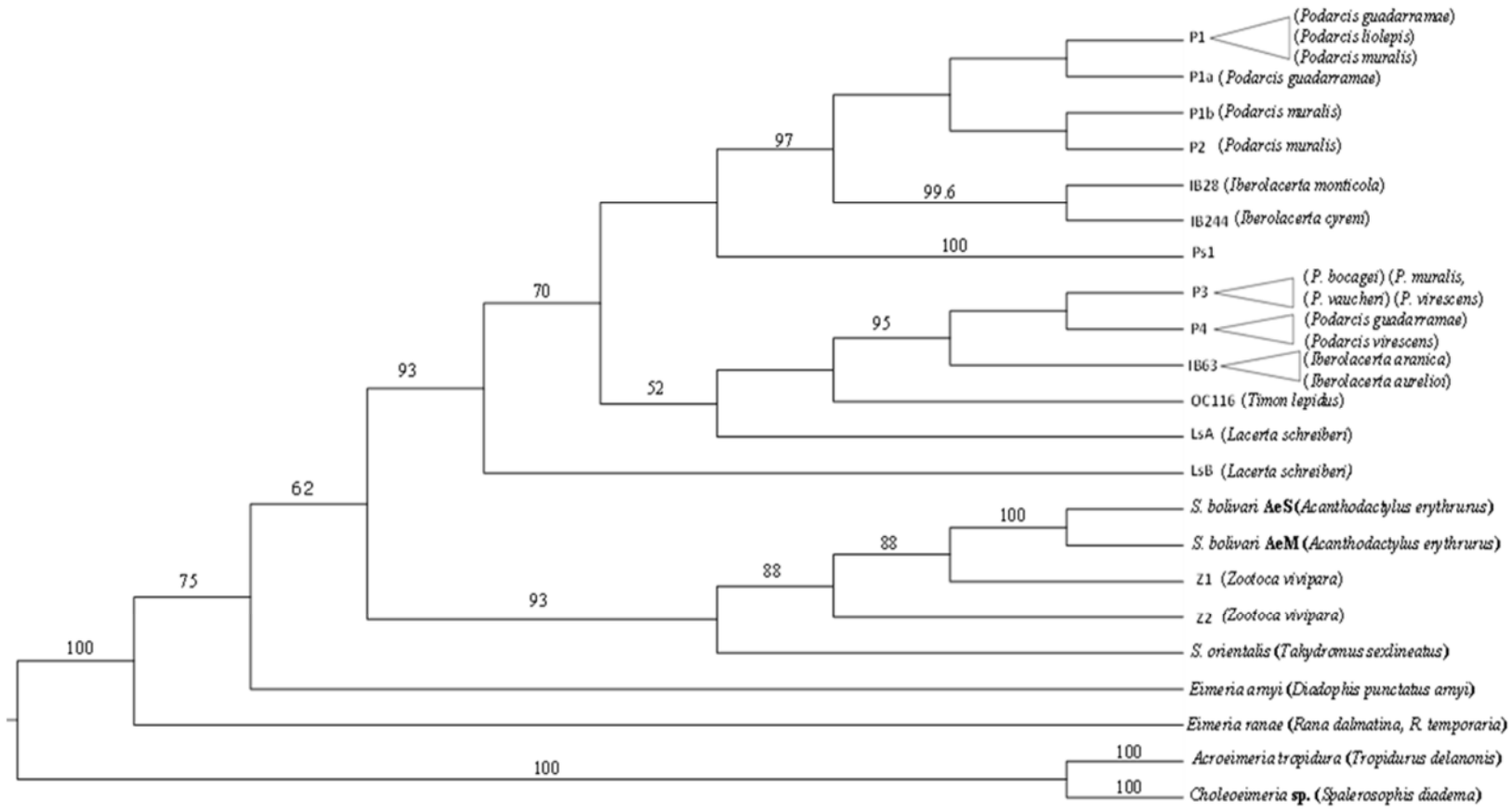

Figure 3. Phylogenetic relationships between *Schellackia* haplotypes in lacertids from the Iberian Peninsula and two localities in the North of Africa based on Bayesian inference. In the terminal nodes appear the *Schellackia* haplotype and the name of the host species where it was found.

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PHYLOGENETIC RELATIONSHIPS OF *ISOSPORA, LANKESTERELLA* AND *CARYOSPORA* SPECIES (APICOMPLEXA: EIMERIIDAE) INFECTING LIZARDS

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Abstract

In this study, several species of *Isospora* infecting lizards were genetically characterized. Specifically, five described and four newly described species of *Isospora* were included in a phylogeny of the family Eimeriidae. These species were isolated from hosts originally inhabiting all geographic continents except Europe. Phylogenetic analyses of the *18S* rRNA gene grouped these nine species of *Isospora* with *Lankesterella* species and *Caryospora ernsti*. Therefore, within this clade, different evolutionary strategies in oocyst development and transmission occurred. Although the characteristic endogenous oocyst development of the genus *Lankesterella* may have arisen only once, the reduction in the number of sporocysts observed in the genus *Caryospora* occurred at least twice during coccidian evolution, as evidenced by the phylogenetic position of *Caryospora bigenetica* as sister taxon of the group formed by reptilian *Isospora*, *Lankesterella* and *C. ernsti*. Within this group, *C. ernsti* was sister taxon to the genus *Lankesterella*. Overall, our results contradict the proposed monophyly of the genus *Caryospora*, highlighting the need for a thorough taxonomic and systematic revision of the group. Furthermore, they suggest that the recent ancestor of the genus *Lankesterella* may have been heteroxenous.

Keywords coccidian · evolution · oocyst · parasite · phylogeny · Squamata

Introduction

The Squamata (Reptilia) have five major genera of Eimeriidae Minchin, 1903 that infect them. These genera are distinguished by the structure of their sporulated oocysts and their life cycles. Specifically, the Squamata host eimeriids with dizoic, tetrasporocyst oocysts that develop on the epithelial surface of the gall bladder or in the microvillous zone of the intestine (i.e. genera *Choeleoeimeria*, *Acroeimeria* and *Eimeria* (i.s.) sensu Paperna and Landsberg, 1989); parasites with single, octozoic sporocyst oocysts with known extraintestinal development, including the formation of fully sporulated oocysts (i.e. genus *Caryospora* Léger, 1904); and parasites with tetrasporozoic, diplosporocystic oocysts (i.e. genus *Isospora* Schneider, 1881). However, the phylogenetic relationships among these groups of parasites remain unknown. In this sense, recent studies have shown that intestinal parasites of the families Lankesterellidae Nöller, 1920 and Schellackiidae Grassé, 1953 with blood stages of transmission in reptile hosts are evolutionarily closely related to genera of the family Eimeriidae (Megía-Palma et al. 2014).

More than 100 species of *Isospora* have been described infecting reptiles around the world, but, to date, none have been molecularly characterized (e.g. Finkelman and Paperna 1994a, b, 1995, 2002; Modrý et al. 1997, 1998, 2004; McQuiston et al. 2001; Abdel-Baki et al. 2013). Therefore, the evolutionary relationships among *Isospora* species infecting reptiles with those infecting birds and mammals are unknown (Carreno et al. 1998; Barta et al. 2005). Here, we molecularly characterized nine *Isospora* species detected in native lizards from four continents. Five of the species correspond to known *Isospora* species, while four are described here for the first time. Furthermore, we molecularly characterized two other apicomplexan parasites isolated from the green anole: *Caryospora ernsti* Upton et al., 1984 and one species of *Lankesterella* Labbé 1899. This study contributes to the unraveling of the phylogenetic relationships between the genera *Isospora*, *Caryospora* and *Lankesterella* infecting lizards.

Materials and Methods

Sample origin and processing

Lizard species in which some isosporoid parasites have already been described were chosen for the present study in order to include described species in the first phylogeny for these reptile-infecting parasites. Furthermore, other Squamata species were also included because they are suspected coccidian hosts, since related species host parasites of the genus *Isospora* and *Caryospora*. The full list of reptile species studied is shown in Table 1a and 1b. In an attempt to include representatives of the genus *Isospora* from all geographic continents containing reptiles, we looked for *Isospora* parasites in potential Iberian host species. To date, no *Isospora* species have been described in endemic Iberian reptiles. To have a broad representation of coccidia in the phylogeny, we also included reptile species belonging to different taxonomic families, namely

Agamidae, Chamaeleonidae, Colubridae, Gekkonidae, Lacertidae, Opluridae, Polychrotidae, Pythonidae, Scincidae, Sphaerodactylidae and Trogonophidae. Some fecal samples were obtained directly from recently imported individuals for sale in pet shops. All fecal samples were collected directly from the cloaca with a standard 1.5 mL vial (Eppendorf Tubes® 3810X, Eppendorf Ibérica, Madrid, Spain) filled with 1 ml of 2% (w/v) potassium dichromate (Duszynski and Wilber 1997). Reptiles were stimulated to defecate by briefly massaging the belly. To enhance the sporulation of coccidian oocysts in the samples, we adapted the protocol described by Duszynski and Wilber (1997). For a week, vials were opened twice a day for 15 minutes each, then closed and vortexed, allowing the air to mix with the sample. After a week, samples were homogenized with a plastic pipette. Some of the sample was taken for microscopic identification of sporulated oocysts. The remaining sample was stored at 4°C for subsequent molecular characterization. We also took blood samples, following the protocol described by Megía-Palma et al. (2013), from 15 green anoles *Anolis carolinensis* Duméril and Bribon, 1837 (Squamata: Polychrotidae) recently imported from the United States by a pet shop.

Microscopic methods

For the microscopic screening of fecal samples, we followed the standard protocol for parasite concentration using the Sheather's sugar flotation technique (Levine 1973). In Table 1, the prevalence (as a percentage) for each surveyed coccidian species is shown. Each sample was screened at 200X magnification with an optic microscope BX41TF (Olympus, Japan). The images used to measure sporulated oocysts of *Isospora* and *Caryospora* and the sporozoites of *Lankesterella* sp. in *A. carolinensis* were taken at 1000X magnification using an adjustable camera on an Olympus SC30 microscope. Always that it was possible, we took at least 20 photographs for each species. Sporulated oocysts and corresponding structures were measured using the MB-Ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/). To compare the size of the oocyst of the species found Canarian lizards (i.e. *Gallotia* and *Tarentola* lizards) we used non-parametric Mann-Whitney *U*-test. For the newly described species, we considered the recommendations of Duszynski and Wilber (1997) and for the description of the morphology of the exogenous oocysts of the new species we attended the standard nomenclature proposed by Berto et al. (2014). The conventional abbreviations for the different oocyst structures were used accordingly. Measurements, including the mean in micrometers, standard deviation and range, of the morphological characteristics of oocysts for each species are given in the taxonomic section and in Table 2.

Molecular methods

We extracted genomic DNA from blood preserved on FTA cards following the protocol described by Megía-Palma et al. (2013). The DNA was then purified using the NZYGelpure kit (NZYTech,

Lda. - genes&enzymes, 1649-038 Lisbon, Portugal). The PowerFecal® DNA Isolation Kit was used to extract DNA from fecal samples (MO BIO Laboratories, Inc. Carlsbad, CA 92010, USA). Partial amplification of the *18S* rRNA gene sequence (1626 bp) was performed using the primers BT-F1 (5´-GGT TGA TCC TGC CAG TAG T-3´) and hep1600R (5´-AAA GGG CAG GGA CGT AAT CGG-3´). These primers were previously used to amplify other coccidian species (see Megía-Palma et al. 2014). Due to the insectivorous diet of some reptilian species, in some fecal samples, we also amplified DNA sequences from haemogregarines found in insects, together with *Isospora*. To avoid this undesired amplification, *Isospora* specific reverse primers, EimIsoR1 (5´-AGG CAT TCC TCG TTG AAG ATT-3´) or EimIsoR3 (5´-GCA TAC TCA CAA GAT TAC CTA G-3´), were used. The size of the amplicons obtained with reverse primers EimIsoR1 and EimIsoR3 were 1580 and 1528 bp, respectively. PCR reactions (total volume of 20 µl) contained between 20 and 100 ng of DNA template. Supreme NZYTaq 2x Green Master Mix (NZYTech, Lda. - genes&enzymes, 1649-038 Lisbon, Portugal) and 250 nM of each primer were generally used. Using a Veriti thermal cycler (Applied Biosystems), reactions were run using the following conditions: 95°C for 10 min (polymerase activation), 40 cycles at 95°C for 30 s, annealing temperature at 58°C for 30 s, 72°C for 120 s and a final extension at 72°C for 10 min.

The 11 DNA sequences (*18S* rRNA) obtained from parasites of lizards were aligned together with 79 other sequences included in a previous study (Megía-Palma et al. 2014). The alignment was performed using PROBCONS (http://toolkit.tuebingen.mpg.de/probcons). Poorly aligned positions and divergent regions of the alignment were removed using GBlocks (Talavera and Castresana 2007) selecting the following options: “Minimum Number of Sequences for a Conserved Position” to 36, “Minimum Number of Sequences for a Flank Position” to 36, “Maximum Number of Contiguous Nonconserved Positions” to 8, “Minimum Length of a Block” to 5 and “Allowed Gap Positions” to “With Half”. The final alignment contained 1500 positions and 90 sequences. The substitution model GTR+I+G was selected using jModeltest 2.1.4 (Darriba et al. 2012) to perform the Bayesian analysis. This analysis consisted of two runs of four chains each, with 5500000 generations per run and a burn-in of 13750 generations (41250 trees for consensus tree). The final standard deviation of the split frequencies was 0.01 in both runs. Convergence was checked using Tracer v1.5 (Rambaut and Drummond 2007). All model parameters were greater than 100.

Table 1a. Reptile species included in this study and the coccidian parasites found in each species. The origin of the reptile species and the microscopic prevalence of the coccidia found are also shown.

Species	Family	N of sampled individuals	Origin	Locality	Coccidian species found	Prevalence of coccidiasis in the sample (%)
Chlamydosaurus kingii	Agamidae	1	Captivity	*Originally from Australia	-	0
Pogona vitticeps	Agamidae	1	Captivity	*Originally from Australia	*Isospora amphiboluri*	100
Chamaleo calyptratus	Chamaeleonidae	1	Captivity	*Originally from Yemen	-	0
Chamaleo melleri	Chamaeleonidae	1	Captivity	*Originally from Africa	-	0
Coronella austriaca	Colubridae	2	Wild	Segovia and Huesca, Spain	-	0
Coronella girondica	Colubridae	2	Wild	Segovia, Spain	-	0
Hemorrhois hippochrepis	Colubridae	1	Wild	Segovia, Spain	-	0
Natrix maura	Colubridae	5	Wild	Segovia, Spain	-	0
Rhinechis scalaris	Colubridae	3	Wild	Segovia, Spain	-	0
Gekko vittatus	Gekkonidae	1	Captivity	Originally from Southeast Asia	-	0
Phelsuma madagascariensis grandis	Gekkonidae	1	Captivity	*Originally from Madagascar	*Isospora gekkonis*	100
Tarentola delalandii	Gekkonidae	2	Wild	Tenerife, Canary Islands	*Isospora tarentolae*	50
Acanthodactylus boskianus	Lacertidae	64	Wild	North Tunisia	*Isospora abdalahi*	10
Acanthodactylus erythrurus belli	Lacertidae	34	Wild	North Morocco	*Isospora fahdi n. sp.*	10
Acanthodactylus erythrurus erythrurus	Lacertidae	24	Wild	Almería, Navarra, Granada, Huelva and Zaragoza, Spain	-	0

Table 1b. Reptile species included in this study and the coccidian parasites found in each species. The origin of the reptile species and the microscopic prevalence of the coccidia found are also shown.

Species	Family	N of sampled individuals	Origin	Locality	Coccidian species found	Prevalence of coccidiasis in the sample (%)
Podarcis bocagei	Lacertidae	10	Wild	León, Spain	-	0
Podarcis hispanica	Lacertidae	10	Wild	Segovia, Spain	-	0
Podarcis muralis	Lacertidae	10	Wild	Segovia, Spain	-	0
Gallotia galloti galloti	Lacertidae	50	Wild	Tenerife, Canary Islands, Spain	*Isospora tarentolae*	6
Iberolacerta cyreni	Lacertidae	40	Wild	Madrid, Spain	-	0
Lacerta schreiberi	Lacertidae	200	Wild	Segovia, Spain	-	0
Psammodromus algirus	Lacertidae	10	Wild	Segovia, Spain	-	0
Takydromus sexlineatus	Lacertidae	13	Captivity	Imported from Indonesia	*Isospora takydromi n. sp.*	23
Timon lepidus	Lacertidae	20	Wild	Segovia, Spain	-	0
Oplurus cyclurus	Opluridae	1	Captivity	*Originally from Madagascar	-	0
Anolis carolinensis	Polychrotidae	15	Captivity	Imported from the USA	*Caryospora ernsti*	20
Anolis carolinensis	Polychrotidae	15	Captivity	Imported from the USA	*Lankesterella sp.*	7
Anolis equestris	Polychrotidae	2	Captivity	Imported from the USA	-	0
Python reticulatus	Pythonidae	10	Captivity	*Originally from Africa	-	0
Chalcides paralellus	Scincidae	13	Wild	Chafarinas Islands, North Africa	*Isospora chafarinensis n. sp.*	46
Chalcides striatus	Scincidae	3	Wild	Segovia, Spain	-	0
Gonatodes albogularis fuscus	Sphaerodactylidae	2	Captivity	Imported from Central America	*Isospora albogulari*	100
Gonatodes ocellatus	Sphaerodactylidae	2	Captivity	*Originally from Central America	-	0
Gonatodes vittatus	Sphaerodactylidae	2	Captivity	*Originally from Central America	-	0
Sphaerodactylus nigropunctatus ocujal	Sphaerodactylidae	2	Captivity	*Originally from Cuba	-	0
Sphaerodactylus notatus	Sphaerodactylidae	2	Captivity	*Originally from Central America	-	0
Sphaerodactylus torrei	Sphaerodactylidae	2	Captivity	*Originally from Cuba	-	0
Trogonophis wiegmanni	Trogonophidae	71	Wild	Chafarinas Islands, North Africa	*Isospora wiegmanniana n. sp.*	52

In addition, the alignment was analyzed using maximum-likelihood inference (PhyML program; Guindon et al. 2010), using the same substitution model mentioned above. The subtree pruning and regrafting (SPR) and the nearest-neighbor interchange (NNI) tree-rearrangements options were selected, and a Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support (Anisimova et al. 2011).

Type photographs and DNA derived from all the material used in this study were deposited in specific collections of the Museo Nacional de Ciencias Naturales-CSIC (Madrid, Spain). The *18S* rRNA gene sequences were deposited in GenBank and are available on request (see Results).

Results

Microscopy and morphology

We found oocysts of nine different *Isospora* species in ten lizard host species belonging to the families Agamidae, Gekkonidae, Lacertidae, Scincidae, Sphaerodactylidae and Trogonophidae from Africa, South America, Asia and Australia (Table 1). Five of the *Isospora* species have been previously described (*Isospora abdallahi* Modrý et al., 1998, *I. albogularis* Upton and Freed, 1990, *I. amphiboluri* McAllister et al., 1995, *I. gekkonis* Upton and Barnard, 1987 and *I. tarentolae* Matuschka and Bannert, 1986). *Isospora tarentolae* was originally described from the Canarian gecko *Tarentola delalandii* Duméril and Bribon, 1836 (Matuschka and Bannert 1986). However, in this study, this parasite was found in two sympatric host species: *T. delalandii* and *Gallotia galloti* Oudart, 1839 (see Figure 1, pictures H and I). Conspecificity was confirmed by both morphology (Mann-Whitney *U*-test: U=14.0, p=0.9 for oocyst length; U= 11.0, p= 0.5 for oocyst width) and molecular analysis of fecal samples that resulted in two sequences 100% coincident.

In addition, we found four new *Isospora* species, which are described in the taxonomic section below. Although we were unable to statistically compare the morphological measures of these species with related ones (the original descriptions lacked some measures, e.g. the standard deviation and/or the number of measured oocysts), the internal structures and general morphology of oocysts were compared.

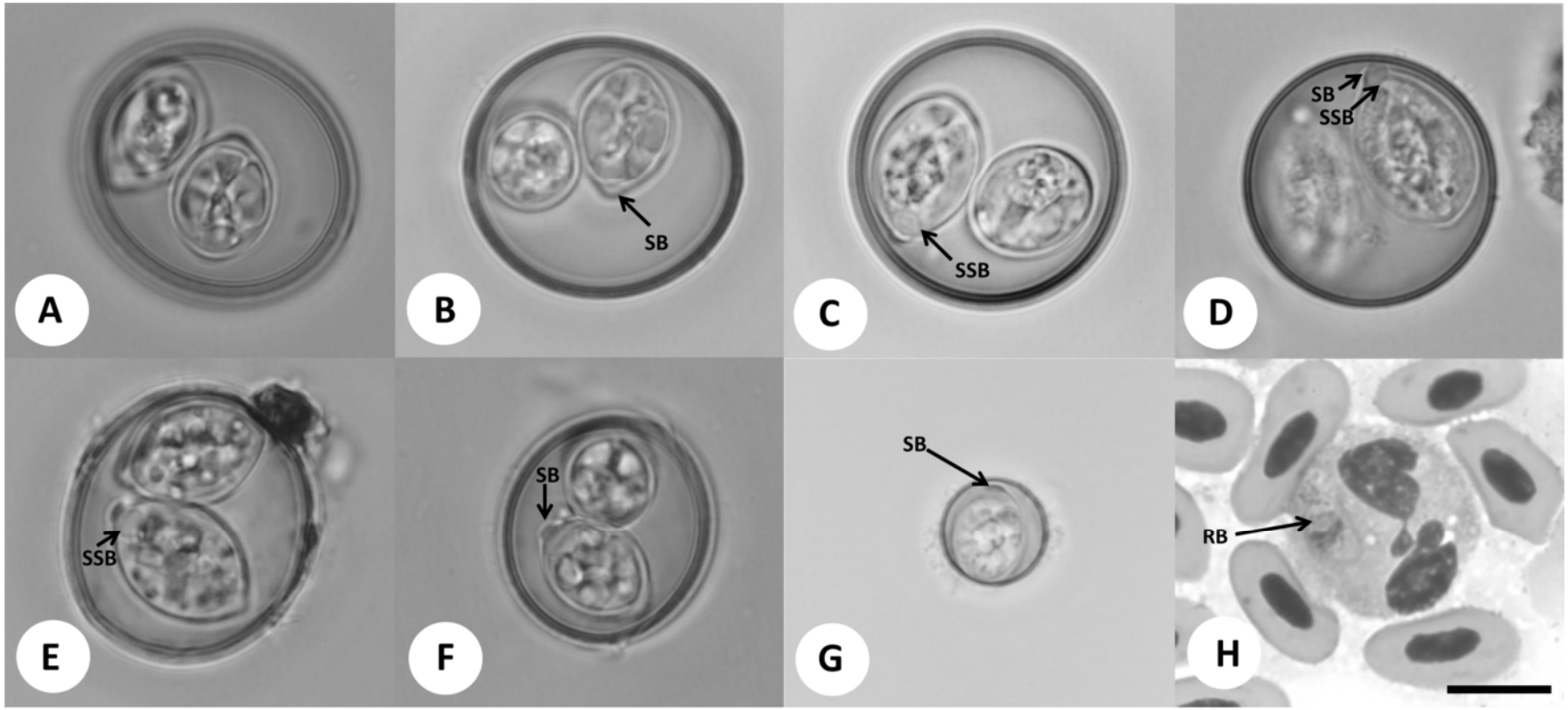


Fig. 1 Infective stages of the different coccidian species found in the present study. All images were taken at the same magnification. Image A-G, exogenous oocysts of coccidian species included in the phylogeny. A. *Isospora tarentolae* from *Tarentola delalandii*. B. *Isospora tarentolae* from *Gallotia galloti.* C. *Isospora abdallahi* from *Acanthodactylus boskianus.* D. *Isospora amphiboluri* from *Pogona vitticeps*. E. *Isospora albogulari* from *Gonatodes albogularis fuscus*. F. *Isospora gekkonis* from *Phelsuma madagascariensis grandis. G. Caryospora ernsti* from *Anolis carolinensis*. H. Sporozoite of *Lankesterella* sp. infecting a polymorphonuclear leukocyte in the blood of *Anolis carolinensis*. SSB: substieda body. SB: Stieda body. RB: refractile body. Scale bar= 10 µm.

Taxonomic section

***Isospora takydromi* sp. nov.** (Figure 2).

Description: The sporulated oocysts (N=26) measured 23.9 ± 3.0 (16.6-30.5) x 19.4 ± 2.3 (24.4-15.3) µm, with a shape index (length/width) of 1.2 ± 0.10 (0.9-1.4). The ellipsoid oocysts had a bilayered wall with a smooth surface. It measured 0.76 (mean) ± 0.1 and ranged from 0.5-1.0 µm thick. There was no micropyle on the surface, and the polar granule (PG) was absent. The tetrasporozoic sporocysts (N=25) were 12.5 ± 1.3 (14.5-9.7) x 8.6 ± 0.6 (9.8-7.5) µm, with a shape index of 1.4 ± 0.1 (1.1-1.6). Specimens presented a flattened knob-like stieda body (SB) on one side of the smooth surface; a rounded substieda body (SSB) was also present (1.5 x 1.0 µm). The sporocyst residuum (SR) was visible among the sporozoites (SP), which were elongated and had two refractile bodies (RB) at either end.
Sporulation: Probably exogenous. The time of sporulation was not recorded.
Type host: *Takydromus sexlineatus* Daudin, 1802.
Origin of the sample: Imported to Spain from Indonesia in 2013. No type locality available.
Prevalence of the parasite: 6/13 (46.1%) of examined individuals were infected.
Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65269. No lizards were euthanized therefore a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: *in process*).
Etymology: The nomen triviale is derived from the generic part of the scientific name of the host, in the genitive singular ending, meaning "of *Takydromus"*. The first parasite species described for a genus of hosts is usually named after the host's generic name. In this case, however, the name was available because the only other species of *Isospora* described in the genus *Takydromus* received the name of the locality where it was discovered (i.e. *I. nagasakiensis* Miyata, 1987).

Taxonomic remark

The size of the oocyst of *I. nagasakiensis* from *T. tachydromoides* Schlegel, 1838 was similar to *I. takydromi* n. sp. (see Table 2). Both species lacked a PG and oocyst residuum (OR) but had a granular SR. However, the exogenous oocyst of *I. takydromi* n. sp. presented a bilayered oocyst wall whereas *I. nagasakiensis* presented a monolayered wall. However, previous evidences suggest that the oocyst wall within Eimeriidae consist of two layers (Belli et al., 2006). Therefore, molecular analyses of *I. nagasakiensis* are needed to compare with *I. takydromi* n. sp. to confirm if they are, in fact, distinct species.

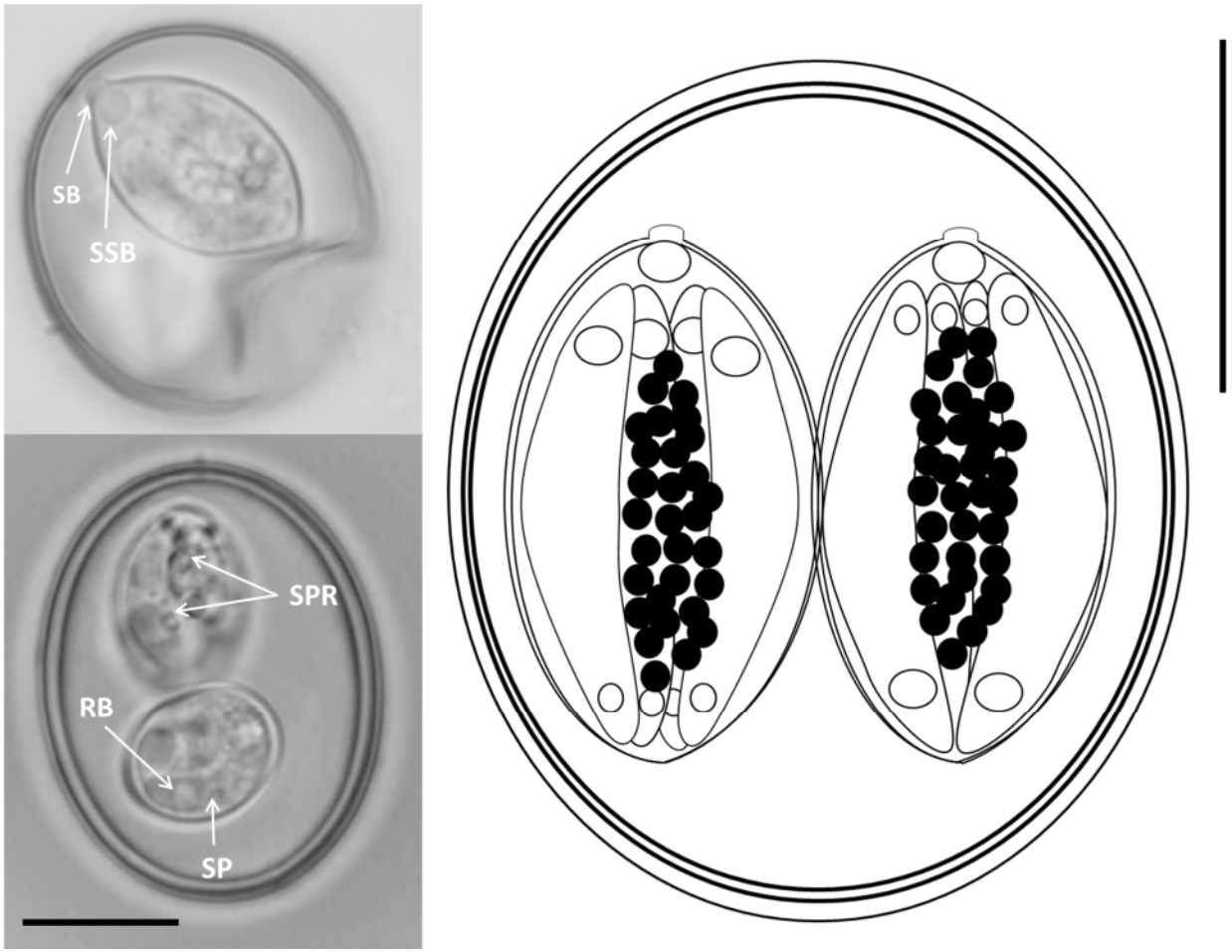


Fig. 2 Microphotographs and line drawing of *Isospora takydromi* n. sp. from *Takydromus sexlineatus* SB: Stieda body. SSB: substieda body. SPR: sporocyst residuum. RB: refractile body. SP: sporozoite. Scale bars= 10 µm.

***Isospora fahdi* sp. nov.** (Figure 3).

Description: The sporulated oocysts (N=28) were subspherical, 25.6 (mean) ± 1.7 (SD) (range= 23.1-29.2) x 22.0 ± 2.2 (18.2-27.1) µm with a shape index (length/width) of 1.17 ± 0.07 (1.01-1.28). The oocyst wall was bilayered with a smooth surface. It measured 1.1 ± 0.1 (0.8-1.3) µm thick. The micropyle, OR and PG were absent. Sporocysts (N=26) were ovoidal, 13.7 ± 1.2 (11.6-16.0) x 9.7 ± 0.6 (8.3-10.9) µm, and had unpigmented and smooth walls. Shape index was 1.4 ± 0.1 (1.1-1.7). The SB was dome-shaped, and the SSB was spherical or subspherical (1.5 x 1.9 µm). The SR was composed of numerous granules of irregular sizes. SP were elongated with distinct anterior and posterior RB.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Acanthodactylus erythurus belli* Grey, 1845.

Type locality: Martil, Tétouan, North Morocco (UTM 30 S 293258, 3946654).

Prevalence: 3/34 (8%) of examined lizards were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65270. No lizards were euthanized therefore a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: *in process*).

Etymology: The specific epithet "fahdi" is a genitive (possessive) Latin name (g. masculine). This patronym (eponym) honors Pr. Dr. Soumia Fahd from the University of Tétouan, Morocco, for her lifelong dedication to herpetological studies of North Africa and in expression of our thanks for her help and hospitality during our field work in Morocco.

Taxonomic remark

The size and morphological characteristics of the oocyst of *I. abdallahi* Modrý et al.,1998 overlap with those of *I. fahdi* n. sp. (see Table 2). However, the molecular data presented here show that the *18S* rRNA gene sequences of *I. abdallahi* and *I. fahdi* n. sp. differ. Therefore, we consider *I. fahdi* a new species based on molecular, and host species differences.

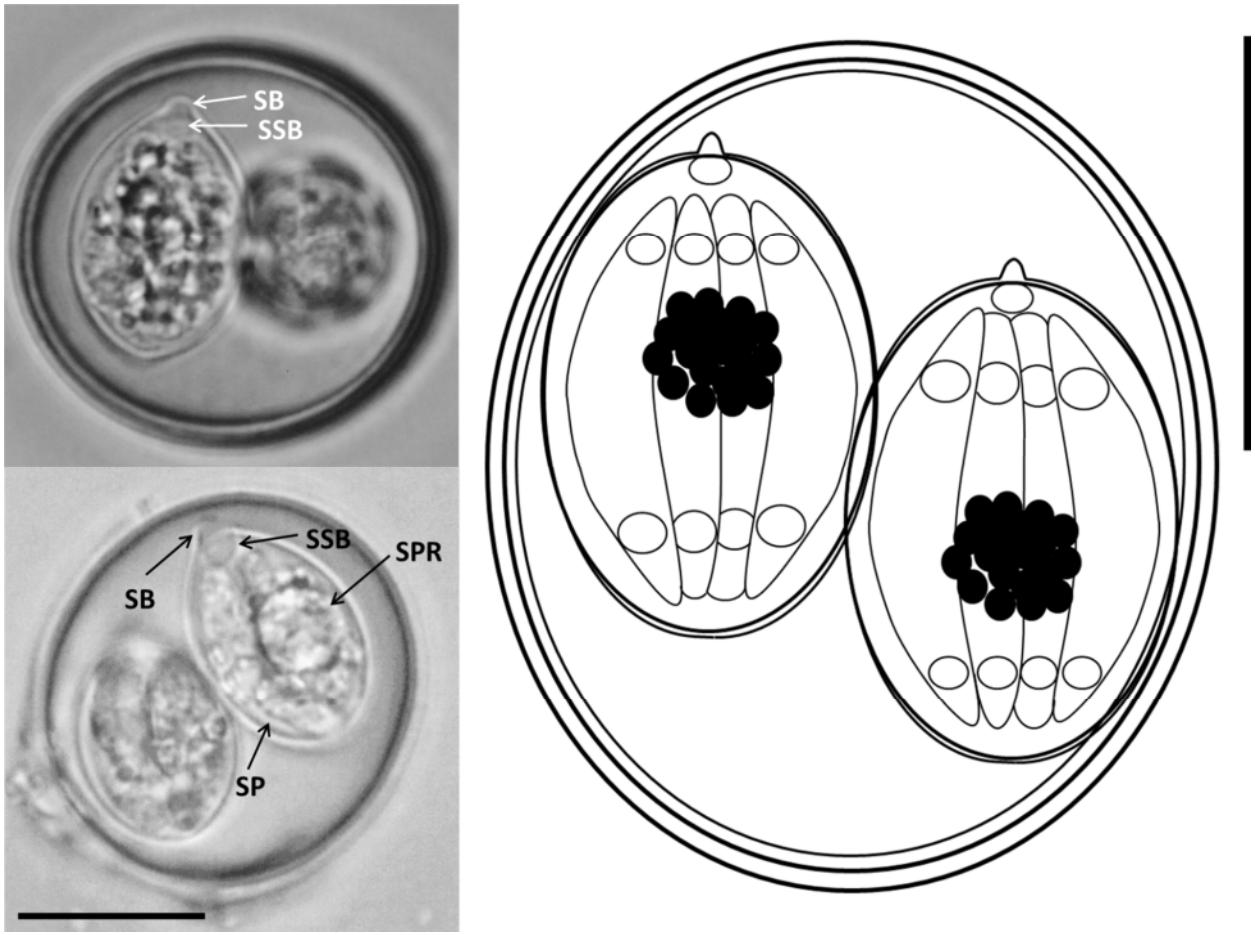


Fig. 3 Microphotographs and line drawing of *Isospora fahdi* n. sp. from *Acanthodactylus erythrurus belli* SB: Stieda body. SSB: substieda body. SPR: sporocyst residuum. SP: Sporozoyte. Scale bars= 10 µm.

***Isospora chafarinensis* sp. nov.** (Figure 4).

Description: The sporulated oocysts (N=62) were subspherical, 21.5 (mean) ± 2.2 (SD) (range= 10.8-24.9) x 20.1 ± 0.9 (17.6-22.0) µm; index shape (length/width) was 1.07 ± 0.10 (0.50-1.20). The micropyle, PG and OR were absent. The sporocysts (N=62) were ellipsoid, 11.6 ± 1.2 (9.3-14.9) x 8.5 ± 0.6 (6.9-9.8) µm; shape index was 1.3 ± 0.1 (1.0-1.8). The SR (N=35) appeared as a granular sphere among the SP and measured 3.7 ± 0.5 (2.4-4.6) µm. A flattened SB and an irregularly rounded SSB were present. A banana-shaped SP had two RB at either end.
Sporulation: Probably exogenous. The time of sporulation was not recorded.
Type host: *Chalcides parallelus* Doumergue, 1901.
Type locality: Rey Francisco Island, Chafarinas Archipelago (Spain), North Africa (UTM 30 S 552523, 3893242).
Prevalence: 6/13 (46.1%) of examined skinks were infected.
Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65272. No lizards were euthanized therefore a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: *in process*).
Etymology: The specific name is a toponymic variable adjective related to the type locality.

Taxonomic remark

Four species of *Isospora* were previously described in the host genus *Chalcides: I. viridanae* Matuschka, 1989, *I. chalchidis* Amoudi, 1989, *I. eimanae* Amoudi, 1989, and *I. arabica* Amoudi, 1993 (see Table 2). The most similar species in size to *I. chafarinensis* n. sp. is *I. viridanae*. Indeed, the oocyst sizes of these species overlap. However, *I. chafarinensis* n. sp. presents sporocysts which are in mean 1.6 µm shorter and 1 µm narrower. Furthermore, there are geographic barriers between the host species: *C. viridanus* Gravenhorst, 1851 is a Canarian endemism in the Atlantic Ocean, whereas *C. parallelus* is a Mediterranean endemism. In addition, the Egyptian species differs in morphology too with *I. chafarienensis* n. sp. The oocyst size of *I. chalchidis* and *I. eimanae* from *C. ocellatus* Forskål, 1775 are respectively 2.6 and 3.1µm shorter in mean to *I. chafarinensis* n. sp. Last, the oocyst of *I. arabica* from the Arabian Peninsula is 11 µm longer and 5 µm wider in mean whereas the sporocyst is 7.4 µm longer and 5 µm wider in mean. *Isospora arabica* has a fairly large SR consisting of diffuse granules whereas *I. chafarinensis* n. sp. presents a granular and dense SR. In addition, *I. chafarinensis* n. sp. is described from Chafarinas infecting *C. parallelus* while *I. arabica* was described from the Arabian Peninsula infecting *C. ocellatus*. Given these morphological, geographic, and host species differences, we consider *I. chafarinensis* as a new species.

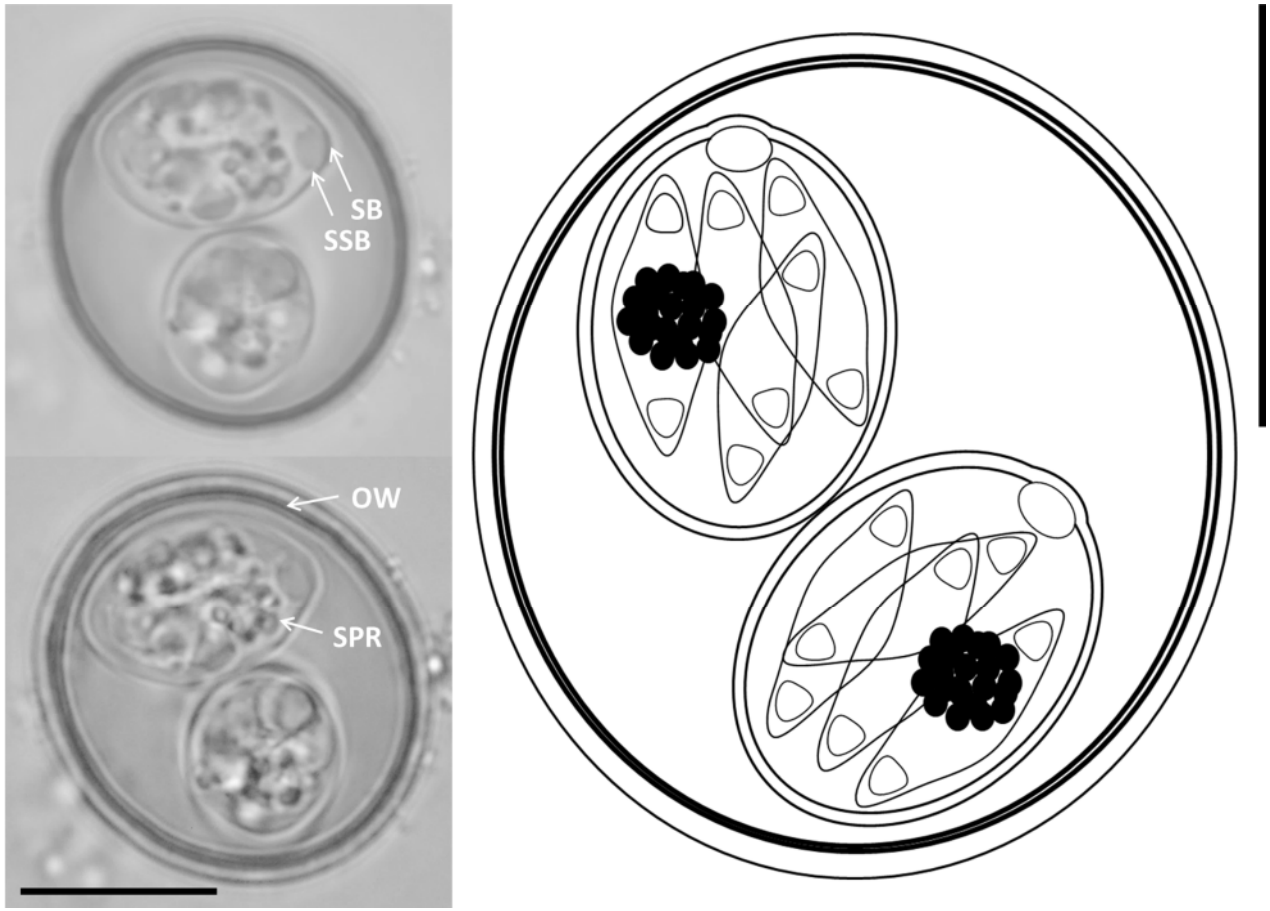


Fig. 4 Microphotographs and line drawing of *Isospora chafarinensis* n. sp. from *Chalcides parallelus*. SB: Stieda body. SSB: substieda body. OW: oocyst wall bilayered. SPR: sporocyst residuum. Scale bars= 10 µm.

***Isospora wiegmanniana* sp. nov.** (Figure 5).

Description: The sporulated oocysts (N=20) were spherical to subspherical, 15.2 (mean) ± 1.0 (SD) (range= 13.1-17.1) x 15.6 ±1.1 (13.9-18.2) µm; index shape (length/width) of 1.04 ± 0.02 (1.01-1.09). Transversal septums were visible in the oocyst wall. A thick monolayered wall of 0.8 ± 0.1 (0.7-1.0) µm was observed. However, there is a growing consensus about the consistency in the structure of the coccidian oocyst wall. Thus likely, two thin or fused layers may form the wall of apparently monolayered walls of coccidian oocysts (Belli et al., 2006; Mai et al., 2009; Berto et al., 2014). The micropyle, PG and OR were absent. Sporocysts (N=20) were ellipsoid, 8.4 ± 1.2 (6.1-10.4) x 6.5 ± 0.5 (5.5-7.6) µm; shape index was 1.2 ± 0.1 (1.0-1.5). An irregular SR, a flattened SB, and a widely flattened SSB were present. Two rounded RB were visible at either end of the SP.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Trogonophis wiegmanni wiegmanni* Kaup, 1830.

Type locality: Congreso, Isabel II and Rey Francisco Islands; Chafarinas Archipelago (Spain), North Africa (UTM 30 S 551837, 3893225).

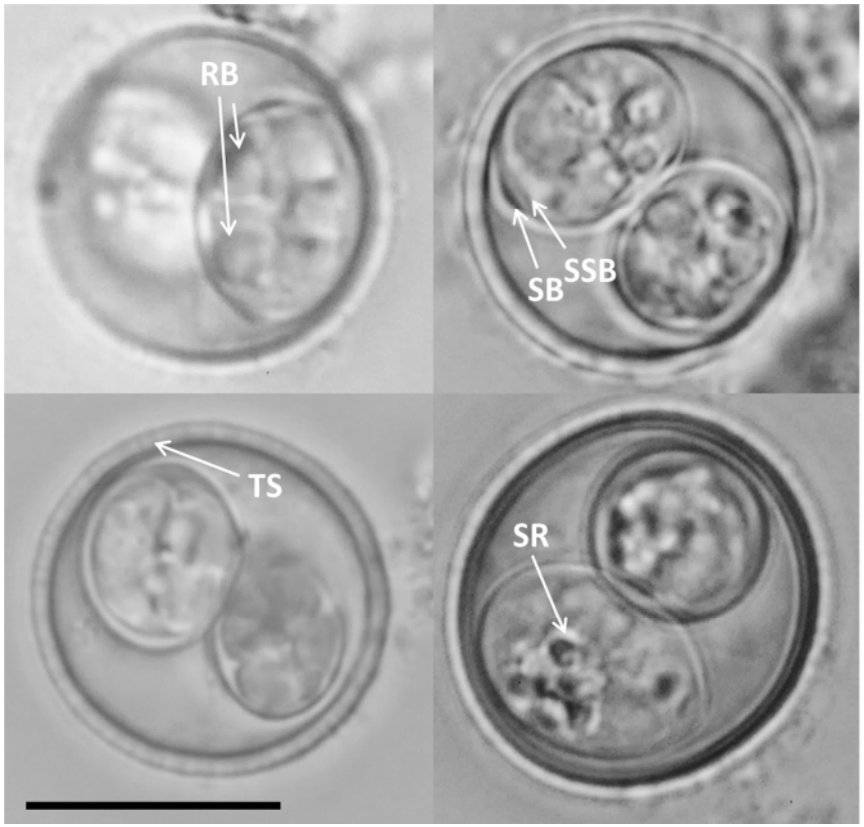
Prevalence: 37/71 (52.1%) of examined amphisbaenians were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65271. No lizards were euthanized therefore a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: *in process*).

Etymology: The nomen triviale was given after the host specific name, and therefore is a variable adjective.

Taxonomic remark

Prior to this study, only one species of *Isospora*, *I. diplometoponi* Al Yousif and Al Shawa, 1998 found in *Diplometodon zarudnyi* Nikolsky, 1907, was known to parasitize the family Trogonophidae. However, this species differs in size from *I. wiegmanniana* n. sp. (see Table 2a and 2b). In addition, contrary to *I. wiegmanniana* n. sp., *I. diplometoponi* has an obvious bilayered oocyst wall with no visible septum and a clearly visible SSB (Al Yousif and Al Shawa 1998). One amphisbaenian species from South America, *I. capanemaensis* Lainson, 2003, is similar to *I. wiegmanniana* in oocyst size. However, in *I. capanemaensis*, the SB is inconspicuous, and the oocyst wall shows no striation (Lainson 2003). Therefore, given the differences in morphology, geographic distribution and host families infected, we propose *I. wiegmanniana* as a new species in the genus *Isospora.* Molecular analyses of these three species are necessary to further support *I. wiegmanniana* n. sp. as a distinct species.



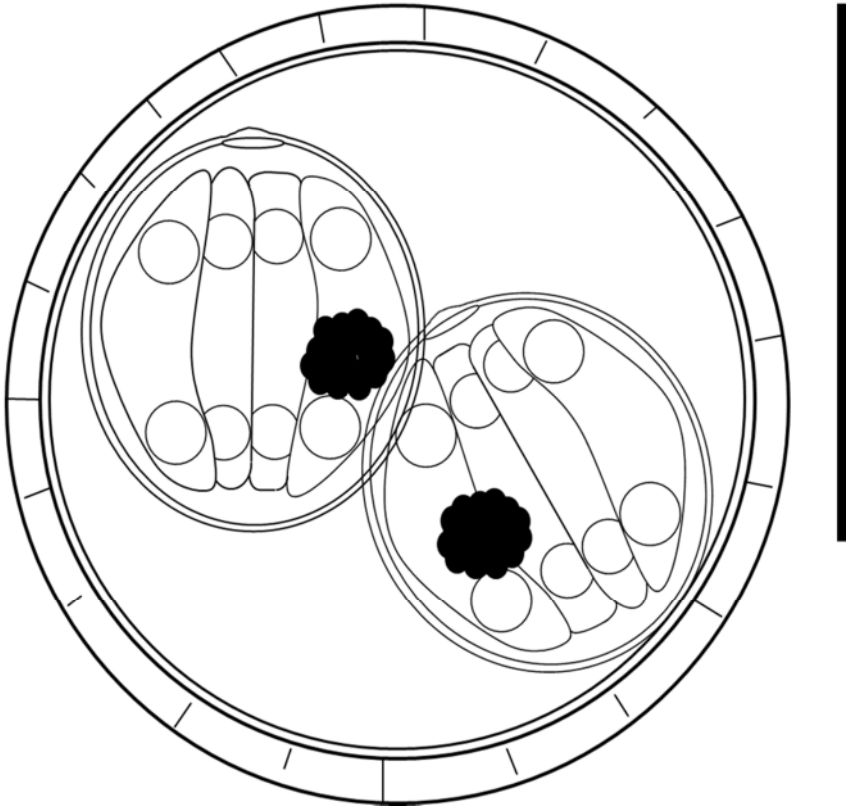


Fig. 5 Microphotographs and line drawing of *Isospora wiegmanniana* n. sp. from *Trogonophis wiegmanni wiegmanni*. RB: refractile body. SB: Stieda body. SSB: substieda body. TS: transveral septums in the wall. SR: sporocyst residuum. Scale bars= 10 µm.

Table 2a. Relevant *Isospora* and *Caryospora* species described from reptiles. *Species included in the phylogeny in the present study.

	Oocyst				Sporocyst						
Species	Length mean	Length range	Width mean	Width range	Length mean	Length range	Width mean	Width range	Host	Locality	Authors
I. abdallahi	25.4	24.5-29.0	23.9	23.0-25.5	15.4	14.0-16.0	9.4	9.0-10.0	*Acanthodactylus boskianus*	Northern Egypt	Modrý et al. 1998
**I. abdallahi*	25.5	22.7-27.9	23.1	20.3-26.1	14.3	11.6-17.0	9.9	9.0-11.4	*Acanthodactylus boskianus*	Tunisia	Present study
I. acanthodactyli	17.2	16.4-18.8	16.4	15.0-17.2	9.3	7.4-10.4	5.9	5.0-6.3	*Acanthodactylus boskianus*	Egypt	Sakran et al. 1994
**I. fahdi* n. sp.	25.6	23.1-29.2	22.0	18.2-27.1	13.7	11.6-16.2	9.7	8.3-10.9	*Acanthodactylus erythrurus belli*	Northern Morocco	Present study
I. acanthodactyli (=I.alyousifi)	27.9	25.1-29.0	25.5	22.7-27.8	11.6	11.2-12.6	8.0	7.5-8.4	*Acanthodactylus schmidti*	Saudi Arabia	Al Yousif & Al-Shawa 1997
I. alyousifi	24.6	17–29	21	16–26	13.5	8–16	9.0	6–11	*Acanthodactylus schmidti*	Saudi Arabia	Abdel-Baki et al. 2012
Caryospora ernsti	12.5	11.0-14.5	12.5	11.0-14.5	10.7	10.0-12.5	8.3	7.5-9.0	*Anolis carolinensis*	United States of America	Upton et al. 1984
**C. ernsti*	12.4	11.4-13.5	12.0	11.0-12.7	9.4	8.5-10.1	7.2	6.3-7.6	*Anolis carolinensis*	Imported from the USA	Present study
C. natchitochesensis	13.1	11.0-15.0	12.3	10.0-14.0	10.1	7.0-13.0	7.4	6.0-10.0	*Anolis carolinensis*	United States of America	McAllister et al. 2014
I.capanemaensis	14.8	13.3-18.0	14.5	12.6-16.3	8.6	7.4-10.4	6.6	5.9-7.4	*Amphisbaena alba*	Capanema, Pará, North Brazil	Lainson 2003

Table 2b. Relevant *Isospora* and *Caryospora* species described from reptiles. *Species included in the phylogeny in the present study.

	Oocyst				Sporocyst						
Species	Length mean	Length range	Width mean	Width range	Length mean	Length range	Width mean	Width range	Host	Locality	Authors
I. chalchidis	19.0	18.0-20.5	19.0	18.0-20.5	12.2	9.5-13.0	6.5	5.0-8.0	*Chalcides ocellatus*	Egypt	Amoudi 1989
I. eimanae	18.5	17.0-19.5	18.5	17.0-19.5	12.0	11.0-13.0	8.5	7.5-9.0	*Chalcides ocellatus*	Egypt	Amoudi 1989
I. arabica	32.5	27.5-34.0	25.0	24.5-26.5	19.0	17.5-21.0	13.5	11.0-14.5	*Chalcides ocellatus*	Saudi Arabia	Amoudi 1993
**I. chafarinensis n. sp.*	21.5	10.8-24.9	20.1	17.6-22.0	11.6	9.3-14.9	8.5	6.9-9.8	*Chalcides parallelus*	Chafarinas Islands (North Africa)	Present study
I. viridanae	21.6	17.6-23.4	-	-	13.2	11.7-14.0	9.5	8.2-10.5	*Chalcides viridanus*	Tenerife, Canary Islands	Matuschka 1989
I. riyadhensis	23.0	18.0-26.0	20.0	17.0-22.0	13.0	11.0-15.0	8.0	7.0-9.0	*Diplometopon zarudnyi*	Central Saudi Arabia	Abdel-Azeem & Al-Quraishy 2011
I. diplometoponi	33.3	28.6-35.2	30.9	26.8-32.7	20.1	17.5-22.3	13.8	12.2-15.4	*Diplometopon zarudnyi*	Eastern Saudi Arabia	Al Yousif & Al Shawa 1998
**I. wiegmanniana* n. sp.	15.7	13.9-18.2	15.2	13.1-17.1	8.4	6.1-10.4	6.6	5.5-7.6	*Trogonophis wiegmanni*	Chafarinas Islands (North Africa)	Present study
I. gallotiae	16.5	15.3-17.6	16.5	15.3-17.6	11.5	10.2-12.2	7.3	5.2-6.6	*Gallotia galloti*	Tenerife, Canary Islands	Matuschka & Bannert 1987
I. albogularis	29.5	26.4-32.0	26.9	22.4-30.8	14.9	13.6-16.0	10.8	10.2-11.4	*Gonatodes albogularis*	Guanacaste, Costa Rica	Upton & Freed 1990

Phylogenetic results

Phylogenetic analysis using the *18S* rRNA gene showed that all nine *Isospora* species found in reptiles are closely related to *Lankesterella* and *Caryospora ernsti* (Figure 3). Within this group, a well-supported monophyletic clade grouped eight of the nine *Isospora* species close to *C. ernsti* and the genus *Lankesterella*. The ninth species, *I. wiegmanniana* n. sp., is the sister taxon to the group compounded by the genus *Lankesterella*, *C. ernsti* and the former eight species of *Isospora*. Furthermore, *Caryospora bigenetica* Wacha and Christiansen, 1982 is sister taxon to the group formed by reptilian *Isospora*, *Lankesterella* and *C. ernsti*. *Lankesterella* obtained from *Anolis carolinensis* grouped with other *Lankesterella* species isolated from *A. erythrurus* Schinz, 1833. These two species are closely related to *L. minima* (Chaussat 1850) Nöller, 1912 and *L. valsainensis* Martínez et al., 2006 isolated from frogs and birds, respectively (Figure 3).

Discussion

Eimeriid coccidia are not expected to be host-specific because it would not be to the parasite's advantage to limit its reproductive opportunities to a single host (Duszynski and Couch 2013). However, *Isospora* species that infect lizards show a high degree of host-specificity evidenced by the high diversity of species described in reptiles (Duszynski, Upton & Couch 2008). The species of *Isospora* isolated from *A. boskianus* Daudin, 1802 and *A. erythrurus belli* are a good example of the host-specificity in this genus. The habitat and distribution of these two phylogenetically closely related host species overlap (Fonseca et al. 2009), but they are parasitized by two different *Isospora* species. This example of host specificity supports the description of new species of coccidian parasites when isolated from different hosts, even when hosts are evolutionarily closely related (e.g. Daszak et al. 2009; Finkelman and Paperna 2002; Modrý et al. 1997, 2004). Therefore, following the criteria of previous studies (e.g. Upton and Barnard 1987; Modrý et al. 1997 and 2004; Modrý and Jirků 2006; Daszak et al. 2009) and given that *T. sexlineatus*, *A. erythrurus belli*, *T. wiegmanni* and *C. parallelus* represent new host species for *Isospora* parasites, we consider these tetrasporozoic, diplosporocystic coccidia as new species of *Isospora*. However, as each host-parasite system has different physiological and immunological peculiarities, molecularly characterizing parasites before describing a new species is desirable.

Supporting this recommendation, we report the occurrence of the same species of *Isospora* in two phylogenetically distant lizards that occupy in sympatry the island of Tenerife (Canary Islands). *Isospora tarentolae* was previously described from the geckonid *T. delalandii* (Matuschka and Bannert 1986). The occurrence of this species in the lacertid *G. galloti* might represent a host-switching event, or alternatively, a case of pseudoparasitism (Ghimire 2010). Previously, other species of *Isospora* were described in more than one host lizard species in islands (Upton and Barnard 1987; Modrý et al. 1997). However, the conspecificity of these

parasites was only based on morphology. In the present case, we could not confirm if the primary host for *I. tarentolae* is the lacertid or the geckonid species because it would have implied to kill the host lizards. However, we hypothesize that *T. delalandii* is the primary host for *I. tarentolae* given the high prevalence of this parasite in *T. delalandii* in this study (50%) and in imported Delalandi's geckoes (60%) from which *I. tarentolae* was originally described (Matuschka and Bannert 1986), together with the low prevalence found in *G. galloti* (6%).

Phylogenetic analyses of isosporoid parasites infecting bird and lizard hosts show the polyphyletic origin of the genus *Isospora* (Barta et al. 2005; Carreno and Barta 1999; Franzen et al. 2000; Frenkel and Smith 2003; Modrý et al. 2001; Morrison et al. 2004). These results emphasize the artificiality of the genus *Isospora* (Modrý et al. 2001), which was described solely based on the number of sporocysts and sporozoites per oocyst and the presence of a SB (Box et al. 1980; Frenkel et al. 1987). Therefore, the common morphological characteristics of the tetrasporozoic, diplosporocystic exogenous oocysts and the presence of a SB in these parasites with separate origins may represent a homoplasy rather than a plesiomorphy (Jirků et al. 2002). The limitations of using morphological or life cycle characteristics for inferring evolutionary relationships among the Eimeriorina have been previously highlighted (Modrý et al. 2001; Barta et al. 2005, Ghimire 2010). For example, the genus *Isospora* (=*Atoxoplasma* Garnham, 1950 pro parte) isolated from birds and the tetrasporozoic, diplosporocystic genera *Besnoitia* Henry, 1913, *Cystoisospora* Frenkel, 1977, *Frenkelia* Biocca, 1968, *Neospora* Dubey et al., 1988, *Sarcocystis* Lankester, 1882 and *Toxoplasma* Nicolle and Manceaux, 1909, all found in mammals, include extra intestinal stages in their life cycles but belong to different families (Eimeriidae and Sarcocystidae Poche, 1913, respectively) (Atkinson et al. 2008; Frenkel and Smith 2003). The independent evolutionary origin of isosporoids from lizards would justify the creation of a new generic name for these parasites. However, despite most of the analyzed *Isospora* species infecting lizards having a recent common ancestor, *I. wiegmanniana* is placed as the sister taxon to the group compounded by *Caryospora*, *Lankesterella*, and the named monophyletic group of *Isospora* suggesting the paraphyletic origin of *Isospora* in lizards (Figure 3). Therefore, it is inappropriate to propose a new generic name for this group (see Morrison 2009).

Similarly, the phylogenetic position of *Caryospora bigenetica* as sister taxon of the group formed by reptilian *Isospora*, *Lankesterella* and *C. ernsti* suggests that the reduction in the number of sporocysts observed in the genus *Caryospora* occurred at least twice during evolution, and that *Caryospora* does not have a monophyletic origin. However, the characteristic endogenous development of oocysts of the genus *Lankesterella* and its transmission by vectors to the next host seem to have arisen only once during evolution in this lineage of parasites. The phylogenetic results here support the polyphyletic origin of the family Lankesterellidae as recently proposed (Megía-Palma et al. 2013, 2014). Therefore, the lack of external oocysts in both *Lankesterella* and *Schellackia* may be a case of convergent evolution, likely driven by behavioral

changes in definitive host species that threatened the successful transmission of the parasite (Barta el al. 2001). These changes in host species may act as evolutionary forces favoring the selection of new parasite transmission strategies. This study reveals, for the first time, the close phylogenetic relationship between the genus *Lankesterella, C.ernsti* and the reptilian *Isospora*.

Figure 6 Phylogenetic tree derived from Bayesian inference using the GTR+I+G substitution model. This analysis consisted of two runs of four chains each, with 5500000 generations per run and a burn-in of 13750 generations (41250 trees for consensus tree). Support values less than 50% are not shown, and these nodes were not collapsed into polytomies. Where two numbers are shown on the branch, the first one indicates the support value obtained by Bayesian inference and the second one by maximum-likelihood (ML) inferences. The ML inference was performed in PhyML also using the GTR+I+G substitution model. Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support. The length of the alignment was 1500 bp

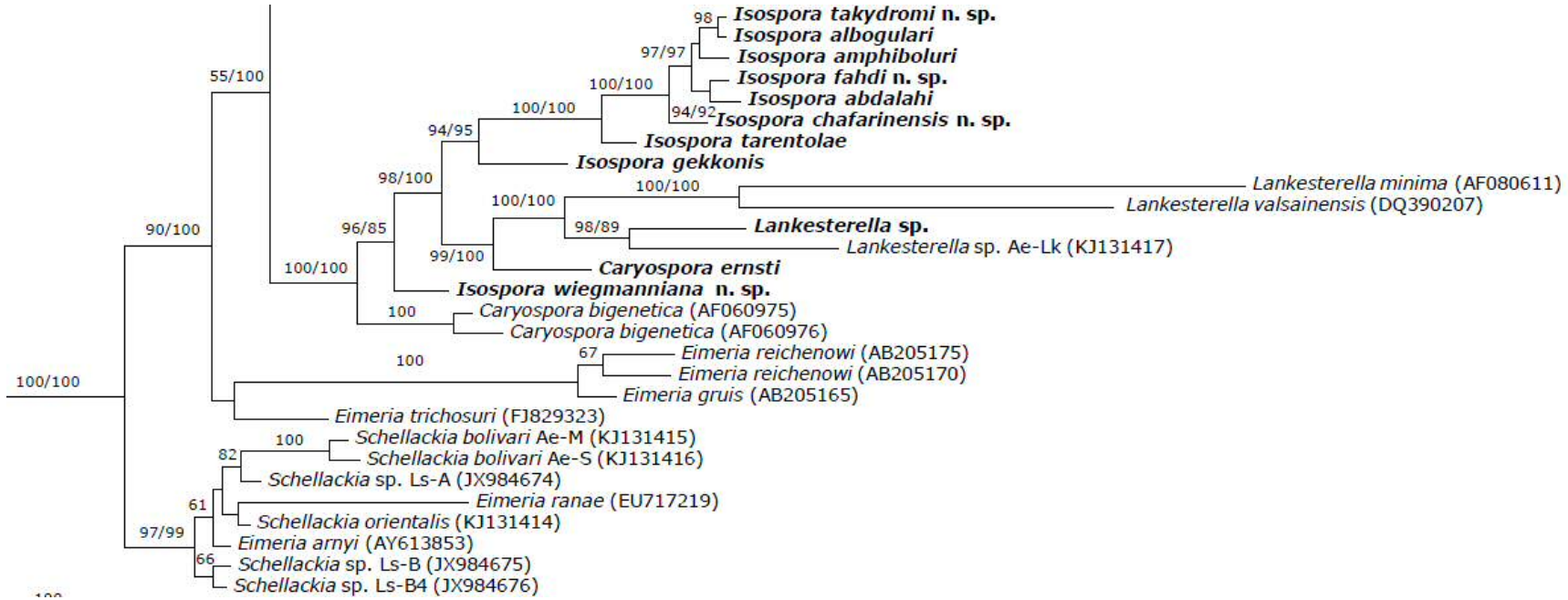


Figure 7. Zoom on the area of interest of the phylogenetic tree of this study. 1) *Caryospora* isolated in lizards is closer related to the genus *Lankesterella* than to *Caryospora* parasites isolated in mice. 2) Isospora-like parasites isolated from fecal boli of lizards are closer related to *Lankesterella* and *Caryospora* parasites than to *Isospora* from passerine birds (see the above tree).

Our results suggest that avian *Lankesterella* species may have evolved from parasites of reptilian hosts and that the recent ancestor of the genus *Lankesterella* may have been heteroxenous. Several studies have shown that some species of *Caryospora* are heteroxenous, with predatory reptiles or birds serving as primary hosts and rodents serving as secondary hosts (Upton et al. 1984, 1986). This variability within the same clade suggests the existence of different selective forces modeling features such as the number of sporocysts per oocyst or the occurrence of endogenous development with naked sporozoites. These changes in developmental stages might lead to species-specific morphological adaptations, as previously suggested for other coccidian parasites (Jirků et al. 2009).

Conclusions

Our results suggest the evolutionary origin of *Isospora* species infecting reptiles is independent from parasites with tetrasporozoic, diplosporocystic oocysts infecting birds, mammals and frogs. They also confirm the artificiality of the genus *Isospora* based on morphological characteristics (see also Modrý et al. 2001). Furthermore, the phylogenetic analysis revealed that the genus *Lankesterella* is closely related to the genera *Caryospora* and *Isospora* found in reptiles. The phylogenetic positions of *C. bigenetica* and *C. ernsti* suggest that the genus *Caryospora* is not monophyletic.

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Conflict of interest The authors declare that they have no conflict of interest.

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PHYLOGENY OF THE REPTILIAN *EIMERIA*: ARE *CHOLEOEIMERIA* AND *ACROEIMERIA* VALID GENERIC NAMES?

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Abstract

Reptiles are the animals with the most described coccidian species among all vertebrates. However, the co-evolutionary relationships in this host-parasite system have been scarcely studied. Paperna & Landsberg (1989) proposed the independent evolutionary origin of the *Eimeria*-like species isolated from reptiles based on morphological and developmental characteristics of their oocysts. Accordingly, they suggested the reclassification of these parasites in two new genera, *Choleoeimeria* and *Acroeimeria*. The validity of the genera proposed to classify reptilian *Eimeria* species remained unresolved due to the lack of species genetically characterized. In the present study, we included *18S* rRNA gene sequences from seven *Eimeria*-like species isolated from five different lizard host families. The phylogenetic analyses confirmed the independent evolutionary origin of the *Eimeria*-like species infecting lizards. Within this group, most species were placed into two monophyletic clades. One of them included the species with ellipsoidal oocysts (i.e. *Choleoeimeria*-like oocysts) whereas the species with more spheroidal oocysts (i.e. *Acroeimeria*-like oocysts) were included in the second one. This result supports the taxonomic validity of the genera *Acroeimeria* and *Choleoeimeria*.

Keywords: eimeriid; Paperna & Landsberg 1989; parasite; protozoa; reptile; specificity; Squamata; systematics.

Introduction

Schneider first described the genus *Eimeria* in 1875 in a rodent species. Since then, about 2,000 species have been described parasitizing both vertebrate and invertebrate hosts (after Upton 2000; Zhao & Duszynski 2001a). However, 98% of *Eimeria* species were described from vertebrate hosts using the characteristic tetrasporocystic, dizoic exogenous oocyst (Asmundsson *et al* 2006; Ghimire 2010). The implementation of molecular techniques rapidly advanced the knowledge of the phylogenetic relationships within the family Eimeriidae (e. g. Zhao & Duszynski 2001a, b; Jirků *et al* 2002; Kvičerová *et al* 2008). In this context, evolutionary trees showed not only the high specificity of these parasites to their vertebrate hosts (Honma *et al* 2007; Power *et al* 2009), but also the paraphyly of the genus *Eimeria* (Morrison 2009). In fact, genera such as *Cyclospora* Schneider 1881, *Caryospora* Léger 1904, *Isospora* Schneider 1881, *Lankesterella* Labbé 1899, and *Schellackia* Reichenow 1919 shared ancestor with *Eimeria* (see Megía-Palma *et al* 2014). Based on previous molecular results (Jirků *et al* 2002) *Eimeria*-like parasites found in reptiles were considered a sister taxon to Eimeriidae and phylogenetically distant from eimeriids isolated from birds and mammals (Jirků *et al* 2002). However, the relationships among the *Eimeria*-like parasites infecting reptiles remained unresolved since only two closely related species were included in the phylogeny of the family (Jirků *et al* 2002, 2009).

Therefore, new species found in reptiles were classified based on characteristic of their life cycles, morphological features of the exogenous oocysts, and the ultrastructure of the different stages of their development (Paperna & Lainson 1999a, b, 2000; Paperna 2003, 2007; Al Nasr 2011). The species infecting reptiles undergo three different types of endogenous development (Lainson & Paperna 1999). On the one hand, parasites with endogenous development occurring in the gall bladder and biliary epithelium surface were proposed to form the genus *Choleoeimeria* (Paperna & Landsberg 1989). On the other hand, eimeriid species developed in the microvillous zone of the intestine might be classified within the genus *Acroeimeria* when the endogenous development is epicytoplasmic, or within the genus *Eimeria* when development is intracytoplasmic (Paperna & Landsberg 1989; Paperna 1994; Lainson & Paperna 1999; Paperna & Lainson 1999b; Modrý & Jirků 2006). Paperna & Landsberg (1989) proposed the genus *Choleoeimeria* including species with an oocyst shape index (OSI; Paperna & Landsberg 1989) threshold of greater than 1.4 (usually 1.6-2.2). The validity of the OSI, in this case, was broadly discussed (Modrý *et al* 2000; Jirků *et al* 2002; Asmundsson *et al* 2006). However, a relationship between OSI value (>1.4) and the location where oocysts undergo the endogenous development (i.e. the gall bladder) is supported for several species (Bovee & Telford 1965a; Asmundsson *et al* 2006). Alternatively, OSI should be less than 1.25 for *Acroeimeria* parasites (Paperna & Landsberg 1989). Other authors preferred to adjust the OSI range for *Choleoeimeria* from 1.5 to 1.8, (but always above 1.4) and commented on the "striking uniformity" of the oocyst morphology within the genus *Choleoeimeria* (Paperna & Landsberg

1989 in Jirků *et al* 2002). Nevertheless, it was pointed out that typically some species of *Eimeria* showing an OSI average around 1.25 have measurement ranges that overlap with those of *Choleoeimeria*. These species could not be classified into any genus before more information became available (Paperna & Landsberg 1989). However, the name *Eimeria incertae sedis (i. s.)* was proposed for those species that did not fit either the amended definition of Eimeriidae (see Jirků *et al* 2002), nor the definition of the genera *Choleoeimeria* or *Acroeimeria* based on the site of their endogenous development (Modrý & Jirků 2006).

Morphological features of the sporocysts were also used to identify eimeriids from poikilotherms. The absence of Stieda body and the presence of alternative opening structures (i.e. bivalve suture) in the sporocysts of these eimeriids (Paperna & Landsberg 1989) were highlighted as indicators of the ancestral origins of this group of parasites (Jirků *et al* 2002, 2009a, b). Based on these features, some authors suggested the resurrection of the family Barrouxiidae sensu Levine (1983) including the genera *Goussia* Labbé 1896, *Choleoeimeria* Paperna & Landsberg 1989 and *Acroeimeria* Paperna & Landsberg 1989 (Berto *et al* 2014). Thus, the presence of the typical suture in the genera *Goussia, Choleoeimeria* and *Acroeimeria* may represent a homoplasy rather than synapomorphy (Jirků *et al* 2002).

There is an open debate about the use of certain characters including singularities of the life cycle and morphometric features of the oocyst to infer the evolutionary relationships among these eimeriids (see Paperna & Landsberg 1989: Lainson & Paperna 1999; Paperna & Lainson 1999b, 2000; Asmundsson *et al* 2006; Modrý & Jirků 2006; Abdel-Baki *et al* 2008; Daszak *et al* 2009). In the present study, we explore the phylogenetic relationships of eimeriid species parasitizing lizards to help clarify the suitability of the genera *Choleoeimeria* and *Acroeimeria*. For this purpose, we use molecular techniques to characterize seven *Eimeria*-like species isolated from five different families of reptiles. We also include the *18*s rRNA gene sequence of other eimeriid species isolated from *Salamandra salamandra* Linnaeus 1758 (Amphibia: Caudata).

Material and methods

Fecal samples were collected from lizard species where various species of the genus *Eimeria* had previously been described. Specifically, we tried to get species belonging to different host families. Fortunately, many of the lizard species known to be hosts for eimerian parasites were available in the pet trade. Apart from those exotic reptiles obtained from pet stores, we primarily sampled reptiles in the field. We obtained samples from the families Gekkonidae, Lacertidae, Phrynosomatidae, Scincidae and Trogonophidae (Table 1).

Table 1. Reptile host species included in this study classified by family, the origin of the samples and the coccidian parasite found.

Species	Common name	Family	Locality	Coccidia found
Gekko gecko	Tokay gecko	Gekkonidae	Pet trade	*Eimeria tokayae*
Tarentola delalandii	Tenerife wall gecko	Gekkonidae	Tenerife, Canary Islands	*Acroeimeria* cf. *tarentolae*
Gallotia galloti	Tenerife lizard	Lacertidae	Tenerife, Canary Islands	*Choleoeimeria gallotiae* n. comb.
Sceloporus occidentalis	Western fence lizard	Phrynosomatidae	Santa Cruz, CA. USA	*Acroeimeria sceloporis*
Salamandra salamandra	Fire salamander	Salamandridae	Monchique, Portugal	*Eimeria steinhausi* n. sp.
Eutropis macularia	Bronze grass skink	Scincidae	Pet trade	*Eimeria* (i. s.) *eutropidis* n. sp.
Mabuya (s. l.) sp.*	Skink	Scincidae	Pet trade	*Choleoeimeria scincorum* n. sp.
Trogonophis wiegmanni	Checkerboard worm lizard	Trogonophidae	Chafarinas Islands	*Choleoeimeria wiegmanniana* n. sp.

*This individual was sampled in a pet store and we lack of accurate information about the host geographic origin and its specific identification.

Additionally, we included a coccidian found in the feces of a Fire Salamander, *S. salamandra* (Caudata: Salamandridae) in order to contribute molecular data from a novel coccidian infection in the order Caudata. In all the cases, the fecal samples were obtained directly from the cloaca of the animals by briefly massaging their belly and collecting them in standard vials filled with 1 ml of 2% (w/v) potassium dichromate to facilitate sporulation (Duszynski & Wilber 1997). In the case of the Fire salamander feces, we tried to aid sporulation of the oocysts by dividing the sample into two parts, one being preserved in tap water (Duszynski & Wilber 1997) and the remaining sample in potassium dichromate. After the process of sporulation, we homogenized the sample using a plastic pipette and used one part of the sample for the microscope identification of the sporulated oocysts. The remaining part of the sample was preserved at 4 °C for later molecular characterization.

Microscopic methods

For the microscopic screening of the samples, we followed the standard protocol of concentration of parasites by means of Sheather's sugar flotation technique (Levine 1973). Each sample was screened at 200X magnification with an optic microscope BX41TF (Olympus, Japan). In order to get representative photomicrographs and to measure the oocysts of the species that we found, we took photos at 400, 600 and 1000X with an adjustable microscope camera (Olympus SC30). Unfortunately, due to the scarce sample from the Canarian geckonid and the salamander we were unable to take pictures at 1000X magnification as is standard for research on eimeriids (Duszynski & Wilber 1997). Therefore, the microphotographs from the exogenous stages were scaled accordingly (Fig. 1) and line drawings of the newly described species were included as supplementary information on line only (Fig. s1). The oocyst shape index was calculated as ratio of the length and the width of each parasite oocyst. Further, the species average OSI was calculated using these data. All measurements from the sporulated oocysts are expressed in micrometers and were taken using the MB-Ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/).

Molecular methods

PowerFecal® DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA 92010, USA) was used to extract DNA from the fecal samples. Thereafter, the DNA was purified using the NZYGelpure kit (NZYTECH, Lda - genes&enzymes). Partial amplification of the *18S* rRNA gene sequence (1,626 bp) was performed using the primers BT-F1 (5´-GGT TGA TCC TGC CAG TAG T-3´)/ hep1600R (5´-AAA GGG CAG GGA CGT AAT CGG-3´). These primers were previously used to amplify other coccidian species (Megía-Palma *et al* 2014). Due to the insectivorous diet of some reptilian species, we amplified haemogregarines together with *Eimeria* in some fecal samples. To avoid this undesired amplification, the specific reverse primers EimIsoR1 (5´-AGG CAT TCC TCG TTG AAG ATT-3´) or EimIsoR3 (5´-GCA TAC TCA CAA GAT TAC CTA G-3´) were designed to substitute for the primer hep1600R. The size of the amplicons obtained with reverse primer EimIsoR1 and EimIsoR3 were 1,580 and 1,528 bp, respectively. PCR reaction volume (20µl) contained between 20 and 100 ng of DNA template. Supreme NZYTaq 2x Green Master Mix (NZYTECH, Lda - genes&enzymes) and 0.25 µM of each primer were routinely used. The reactions were cycled under the following conditions using the Verity thermal cycler (Applied Biosystems): 95 °C for 10 min (polymerase activation), 40 cycles at 95 °C for 30 s, annealing temperature for 58 °C for 30 s, 72 °C for 120 s and a final extension at 72 °C for 10 min.

The eight DNA sequences (*18*S rRNA) obtained in the present study were aligned together with 79 other sequences included in a previous study (Megia-Palma *et al* 2014). The alignment was performed using PROBCONS (http://toolkit.tuebingen.mpg.de/probcons). Poorly aligned positions and divergent regions of the alignment were suppressed using GBlocks program (Talavera & Castresana 2007) selecting the following options: “Minimum Number of Sequences for a Conserved Position” to 44, “Minimum Number of Sequences for a Flank Position” to 44, “Maximum Number of Contiguous Nonconserved Positions” to 8, “Minimum Length of a Block” to 5, and “Allowed Gap Positions” to “With Half”. The final alignment contained 1,527 positions and 86 sequences. The substitution model GTR+I+G was selected using jModeltest 2.1.4 (Darriba *et al* 2012) to perform the Bayesian analysis. This analysis consisted of 2 runs of 4 chains each, with 5,000,000 generations per run and a burn-in of 1,250,000 generations (37,500 trees for consensus tree). The final standard deviation of the split frequencies was 0.01 in both analyses. Convergence was checked using the Tracer v1.5 software (Rambaut & Drummond 2007). All of the model parameters exceeded 100.

In addition, the alignment was analyzed using the maximum-likelihood inference (PhyML program; Guindon *et al* 2010). The substitution models were those indicated above, the subtree pruning and regrafting (SPR) and the nearest-neighbor interchange (NNI) tree-rearrangements were selected, and a Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade

support (Anisimova *et al* 2011).

Results

Microscopy results

We found oocysts of eimerian parasites in seven lizard species and one Fire salamander. Three of these host species were sampled in captivity and five of them in the field (Table 1). Three of the coccidian species were already described (*Eimeria gallotiae* Matuschka & Bannert 1987, *E. tokayae* Ball & Daszak 1995 and *Acroeimeria sceloporis* (Bovee & Telford 1965b) Paperna & Landsberg 1989). However, the endogenous development was only known for *A. sceloporis* Paperna & Landsberg 1989 (see Bovee & Telford 1965b). In the supplementary information (on-line), we describe four new species of *Eimeria*-like parasites found in lizard hosts, we re-described *E. gallotiae* Matuschka & Bannert 1987, *E. tropidura* Aquino-Shuster, Duszynski & Snell 1990, and *Eimeria* cf. *tarentolae* Matuschka & Bannert 1986 and we describe a new species of *Eimeria*-like parasite found in Caudata hosts.

Phylogenetic results

All eimeriid species isolated from reptilian hosts, except *E. arnyi* Upton & Oppert 1991, form a well-supported monophyletic group (Fig. 2). This clade presented a basal position with respect to the rest of *Eimeria* species except *E. steinhausi* n. sp. Within this group of *Eimeria*-like parasites of reptiles, we found a strongly supported group with oocyst morphology consistent with *Acroeimeria*. *Acroeimeria sceloporis* was the sister taxa to *A. tropidura* n. comb. Both taxa were found in American lizards (Aquino-Shuster *et al* 1990; Bovee & Telford 1965b). These two *18*s rRNA gene sequences were closely related to that from *A.* cf. *tarentolae* n. comb. found in *Tarentola delalandii* Duméril & Bribon 1836 from the Canary Islands.

On the other hand, we found four sequences of *Eimeria*-like species with oocyst morphology consistent with *Choleoeimeria* sensu Paperna & Landsberg 1989 (i.e. *Choleoeimeria* sp. 1, *C. gallotiae* n. comb., *C. wiegmanniana* n. sp. and *C. scincorum* n. sp.). These sequences formed a strongly supported clade and were also closely related to a species with a rounded oocyst, i.e. *Eimeria* (i. s.) *eutropidis* n. sp. In relation with *E. steinhausi* n. sp. found in *S. salamandra,* the topology of the tree showed its ancestral origin in comparison with the rest of Eimeriidae species, including *Goussia* spp. from anuran and fish hosts. However, this relationship was moderately supported in the phylogeny (see Fig. 2).

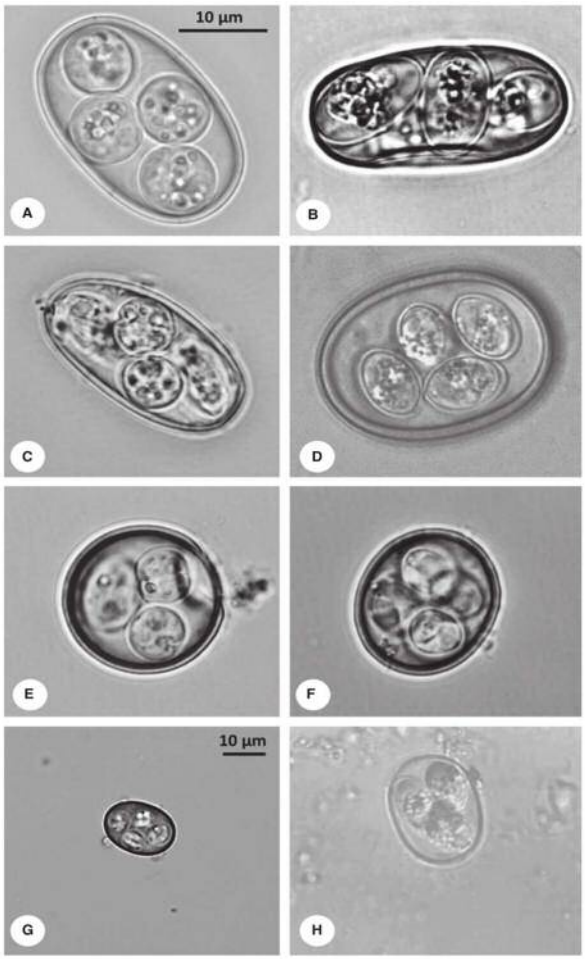

Figure 1. A-H, exogenous oocysts of the *Eimeria*-like species found in the reptile hosts included in the phylogeny of this study. **A-F** and **G-H** are shown at the same scale. **A**. *Choleoeimeria wiegmanniana* n. sp. from *Trogonophis wiegmanni* (Trogonophidae). **B**. *Choleoeimeria gallotiae* n. comb. from *Gallotia galloti* (Lacertidae). **C**. *Choleoeimeria scincorum* n. sp. from *Mabuya* (s. l.) sp. **D**. *Acroeimeria sceloporis* from *Sceloporus occidentalis* (Phrynosomatidae). **E**. *Eimeria tokayae* from *Gekko gecko* (Gekkonidae). **F**. *Eimeria* (i. s.) *eutropidis* n. sp. from *Eutropis macularia* (Scincidae). **G**. *Acroeimeria* cf. *tarentolae* n. comb. from *Tarentola delalandii* (Gekkonidae). **H**. *Eimeria steinhausi* n. sp. from *Salamandra salamandra* (Caudata: Salamandridae).

Discussion

Based on characteristics of internal and external stages or the phylogenetic relationships studied thus far, the evolutionary origin of the *Eimeria*-like species that infect reptiles was considered independent from that of other eimeriids found in mammals and birds (Jirků *et al* 2002; Paperna 2007; Jirků *et al* 2009a, b). In fact, all the species included in the present study grouped in a reptile-specific clade that supports the hypothesis of separate originations of these parasites. Within this clade, the species with OSI~1.3 and OSI> 1.4 grouped with morphological consistency.

Figure 2. Phylogenetic tree showing the evolutionary relationships among the Eimeriorina. The Bayesian inference used the GTR+G+I substitution model. This analysis consisted of two runs of four chains each, with 5,000,000 generations per run and a burn-in of 1,250,000 generations (37,500 trees for consensus tree). All branches were maintained but support values less than 50% were suppressed. Where two numbers are shown in the branch, the first one indicates the supporting value achieved by Bayesian inference and the second one by maximum-likelihood inferences (ML). The ML inference was performed using PhyML program selecting the GTR+I+G substitution model. Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support. The length of the alignment was 1,527 pb.

Acroeimeria tropidura n. comb., *A. sceloporis* and *A.* cf. *tarentolae* n. comb. with OSI~1.3 grouped together. The high morphological and phylogenetic consistency (see Fig. 2) of this clade supports the monophyly and therefore the validity of the genus *Acroeimeria* sensu Paperna & Landsberg (1989). Nevertheless, *A.* cf. *tarentolae* n. comb. separated first from *A. sceloporis* and *A. tropidura* n. comb. (see Fig. 2) and, therefore, the endogenous development of this species should be studied to confirm its consistency with *Acroeimeria* (Paperna & Landsberg 1989).

The four *Eimeria*-like species whose oocysts exhibited an OSI>1.4 formed a well-supported clade (see Fig. 2). If the morphology of the oocyst is related to site of endogenous development in the host, the three species with OSI> 1.4 included in the phylogenetic analyses may develop in the host's gall bladder and the biliary epithelium (Bovee & Telford 1965a; Paperna & Landsberg 1989; Daszak & Ball 1991; Jirků *et al* 2002; Asmundsson *et al* 2006). The morphological consistency of the oocyst and the phylogenetic relationship of these species lend validity to the genus *Choleoeimeria.* In addition, the evolutionary tree indicated a recent origin of these *Choleoeimeria* species compared with its sister taxon, *Eimeria* (i. s.) *eutropidis*, which show an OSI of ~1.0. This morphometric feature could suggest that the ancestor of *Choleoeimeria* may resemble an *Eimeria*-like parasite with rounded oocysts and intestinal development. Thus, the ellipsoidal oocysts could be an adaptation to the physiognomy of the host's gall bladder. Alternatively, the spherical oocysts of *Eimeria (i. s.) eutropidis* could develop in the gall bladder indicating that this developmental characteristic would not be a synapomorphic character for *Choleoeimeria*. It is clearly necessary to investigate the endogenous development of the species with conflicting phylogenetic positions to confirm if the morphology of the oocyst is related to the location of the endogenous development in the host (Paperna & Landsberg 1989). In this sense, the uncertain phylogenetic position of *E. tokayae* along with its oocyst morphology with an OSI~1.0 prompted us to include it within the *Eimeria* incertae sedis sensu Paperna & Landsberg (1989).

The designation of separate genera with different monophyletic clades within Eimeriidae was encouraged by previous studies (Morrison 2009; Ghimire 2010). Therefore, we consider that the use of the genera *Acroeimeria* and *Choleoeimeria* sensu Paperna & Landsberg 1989 is justified even though we do not know their endogenous development. In fact, in previous studies of *Eimeria*-like parasites of reptiles the morphology of the oocyst was related with the location of the endogenous development in the host's tissues (Bovee & Telford 1965a, b; Paperna & Landsberg 1989; Ball & Daszak 1995; Lainson & Paperna 1999; Paperna & Lainson 2000; Asmundsson *et al* 2006; Paperna 2007; Al-Quraishy 2011; Abdel-Baki *et al* 2013). Moreover, the use of sequencing data to determine other coccidian species without obtaining the characteristics of endogenous oocysts was implemented before in the genera *Schellackia* and *Lankesterella* (Merino *et al* 2006; Biedrzycka *et al* 2013; Megía-Palma *et al* 2013, 2014). This method is

particularly useful to avoid killing the reptile hosts, because it would concern conservation and ethical issues.

In regard to the family Eimeriidae, the tree indicates an evolutionarily ancestral position of the group formed by the *Eimeria*-like species from reptiles, in relation to other taxa in the family (excluding those of *Salamandra*). However, the phylogenetic position of this coccidian species isolated from Caudata hosts was poorly supported. The phylogenetic analyses showed that *Goussia* evolved independently from the *Eimeria*-like species found in reptiles (Jirků *et al* 2009b) rejecting a recent hypothesis that placed both taxa under the family Barrouxidae (Berto *et al* 2014). However, the oocysts of both groups shared morphological characteristics such as the occurrence of bivalved sporocysts (Jirků *et al* 2002). The occurrence of this feature might be a plesiomorphy shared by *Eimeria*-like species from poikilotherm hosts (amphibia, reptile, fish, and invertebrate hosts). This characteristic also suggested an early evolution of the parasitic relationships between eimeriid coccidia and cold-blooded vertebrate hosts. Though *Eimeria arnyi* and *E. ranae* are exceptions to this rule. They are *Eimeria*-like species, parasitizing reptilian *(Diadophis punctatus arnyi)* and anuran hosts *(Rana temporaria)*, closely related to *Schellackia* species. However, it is necessary to resample data from these parasites to discard a hypothetical misidentification (Megia-Palma *et al* 2014). Later on, evolutionary radiation of the family Eimeriidae could occur due to the emergence of the Stieda body. This structure located in sporocysts is an apomorphic trait of *Eimeria* sensu lato and it might confer a preadaptation to parasitizing other groups of vertebrates (Jirků *et al* 2009b).

Three of the samples that were included in the present study were obtained from reptiles kept in captivity. The risk of pseudoparasitization due to the inespecificity within Eimeriidae was reported before (see Ghimire 2010). Therefore, the parasites found in the pet trade might result in misidentification of their proper host species. However, in the present study we selected reptile stores where the lizard species were housed separately to minimize the chances of cross-infection. Furthermore, we were able to find other coccidian species in neighbor terraria containing different host species (Megía-Palma *et al* in preparation) but we never found cross-infections either by microscopy or by molecular tools in different host species. This is not the first time that parasites have been described from reptile hosts in captivity evidencing the suitability of using imported species to detect indigenous parasites (e. g. McAllister *et al* 1995, 2014; Megía-Palma *et al* 2014). Moreover, the phylogenetic position, the morphology of the oocyst, and the high number of oocysts (R. Megía-Palma pers. obs.) of the *Eimeria*-like species found in the two species of skink and the Tokay gecko support this argument.

In conclusion, the reptilian *Eimeria* species form a well-supported monophyletic clade and the use of the genera *Choleoeimeria* and *Acroeimeria* proposed by Paperna & Landsberg in 1989 seems to be justified from both a morphological and phylogenetic point of view.

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Supplementary information

Taxonomic section

***Choleoeimeria wiegmanniana* sp. nov.** (Figure 1, microphotograph A; Figure s1, A).

Description: The sporulated oocysts (N=22) are cylindroidal with a mean length and standard deviation (SD) of 28.7 ± 1.3, ranging from 26.1 to 31.2 and a mean width and SD of 18.9 ± 0.6 (17.7-20.1) µm; oocyst shape index (OSI; length/width) of 1.5 ± 0.07 (1.3-1.6). The bilayered oocyst wall is 1.05 ± 0.1 (0.8-1.2) µm thick. Micropyle and oocyst residuum (OR) are absent. A spherical polar granule (PG) is present of 1.1 µm. The four sporocysts (SP) present in the sporulated oocyst are spherical, 8.7 ± 0.5 (7.7-9.8) x 7.9 ± 0.4 (7.2-8.9) µm; sporocyst shape index (SSI) 1.09 ± 0.06 (1.03-1.2). Sporocyst residuum (SR) is present, no Stieda (SB) or substieda bodies (SSB) are observed. There are two sporozoites (SP) per sporocyst.

Sporulation: To the time of the observation of the samples, the oocysts were sporulated. The time of sporulation was not recorded.

Type host: Checkerboard worm lizard, *Trogonophis wiegmanni wiegmanni* Kaup, 1830.

Type locality: Congreso, Isabel and Rey; Chafarinas Islands (Spain), North Africa (UTM 30 S 551837, 3893225).

Prevalence: 38/71 (53.5%) examined worm lizards were infected.

Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de

Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85538. No lizard was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360733).

Etymology: The nomen triviale was given after the host specific name, and therefore is a variable adjective.

Taxonomic remark: This is the second *Eimeria*-like species reported from worm lizards of the family Trogonophidae so far. Although there are four species of *Eimeria*-like parasites that were found infecting amphisbaenians (see Table s1) three of them were found in amphisbaenians from America and the size of the oocyst are fairly distinguishable. The most similar species to *C. wiegmanniana* n. sp. is *C. zarudnyi* (Alyousif and Al-Shawa 2003) Abdel-Baki, Abdel-Haleem & Al-Quraishy 2013. However, the size of the sporocysts is smaller in *C. wiegmanniana* n. sp. Furthermore, *C. wiegmanniana* n. sp. presents a spherical PG and a SR which is absent in *C. zarudnyi.*

***Choleoeimeria scincorum* sp. nov.** (Figure 1, photomicrograph C; Figure s1, B).

Description: The sporulated oocysts (N= 14) are cylindroidal with a mean length and SD of 27.2 ± 1.2 (25-29.6) µm and a mean width of 14.0 ± 0.7 (12.4-15.2) µm; OSI of 1.94 ± 0.1 (1.7-2.2). The bilayered oocyst wall consisted of an inner layer of approximately 0.4 µm and an outer layer of approximately 0.5 µm thick. Micropyle and OR are absent. An elongated PG of approximately 1.7 x 1.1 µm is observed occasionally. The four SP present in the sporulated oocyst are ovoidal, (N= 14) 8.8 ± 0.9 (7.2-10.1) µm x 7.0 ± 0.4 (6.2-7.5) µm; SSI 1.2 ± 0.1 (1.0-1.4). Sporocyst residuum (SR) is present of approximately 4.0 µm, no Stieda (SB) or substieda bodies (SSB) are observed. There are two sporozoites (SP) per sporocyst.

Sporulation: To the time of the observation of the samples, the oocysts were sporulated. The time of sporulation was not recorded.

Type host: Skink, *Mabuya* (s. l.) sp. Fitzinger 1826.

Type locality: Imported lizard from unknown origin.

Prevalence: 1/1 (100%) examined lizard was infected.

Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85539. No lizard was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360730).

Etymology: The nomen triviale means in relation to Scincidae, and therefore is a variable

adjective.

Taxonomic remark: The oocyst size of *C. jazanensis* Abdel-Baki, Al-Quraishy & Abdel-Haleem 2013 found in *Scincus hemprichii* Wiegmann 1837 overlap with *C. scincorum* n. sp. (see Table s2). However, the second present a clearly distinguishable bilayered oocyst wall, and the sporocyst of *C. scincorum* n. sp. was in mean 4 µm shorter. Either species present an OR but *C. scincorum* n. sp. showed, in addition, an elongated PG.

***Eimeria (i. s.) eutropidis* sp. nov.** (Figure 1, photomicrograph F; Figure s1, C).

Description: The sporulated oocysts (N= 21) are spherical or subspherical 14.0 (mean) ± 0.6 (SD) (range= 12.9-15.2) x 13.4 ± 0.6 (12.2-14.7) µm; OSI of 1.0 ± 0.04 (0.9-1.1). The bilayered oocyst wall is 0.9 ± 0.1 (0.6-1.2) µm thick. Micropyle, PG and OR are absent. The sporulated oocyst contains four ellipsoidal sporocysts, (N= 21) 7.0 ± 0.8 (5.2-8.4) x 5.1 ± 0.5 (4.3-5.9) µm; shape index 1.3 ± 0.1 (1-1.6). SR absent, no SB or SSB are observed. There are two SP per sporocyst.

Sporulation: To the time of the observation of the samples, the oocysts were sporulated. The time of sporulation was not recorded.

Type host: Bronze grass skink, *Eutropis macularia* Blyth, 1853.

Type locality: Imported animals from Thailand.

Prevalence: 5/9 (55.5%) examined lizard were infected.

Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85540. No lizard was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360729).

Etymology: The nomen triviale was given after the generic name of the host meaning "of *Eutropis*". It is a Latin genitive thus, not variable.

Taxonomic remark: The most similar species to *E.* (i. s.) *eutropidis* n. sp. is *E. minetti* Ray, Raghavarchari and Sapre 1942 from India (Table s2). Although the current distribution of the lizard host of *E.* (i. s.) *eutropidis* n. sp. includes India, the morphology of the oocysts and sporocysts of *E. minetti* fairly differed from *E.* (i. s.) *eutropidis* n. sp. The oocyst of *E.* (i. s.) *eutropidis* n. sp. is spherical to subspherical whereas in *E. minetti* the oocyst is ovoid. Furthermore, the sporocyst of the new species is 2 µm shorter and narrower in mean.

***Eimeria steinhausi* sp. nov.** (Figure 1, photomicrograph H; Figure s1, D).

Description: The sporulated oocysts (N= 5) are oval 26.9 ± 1.2 (25.0-28.4) µm x 21.5 ± 0.6 (20.6-22.1) µm; OSI of 1.2 ± 0.08 (1.1-1.3). The bilayered wall consists of an inner and outer layer of approximately 0.5 µm thick each. Micropyle, PG and OR are absent. The sporulated oocyst contains four ellipsoidal sporocysts (N= 5), 12.4 ± 0.5 (11.6-13.1) µm x 8.2 ± 0.4 (7.7-8.9) µm. SR absent, no SB or SSB are observed. There are two SP per sporocyst.

Sporulation: Approximately 2% of the oocysts were sporulated at the time the sample was observed, only 5 oocysts were found completely sporulated. Any better result was obtained by including the samples from the Fire salamander in potassium dichromate or tap water.

Type host: Fire salamander, *Salamandra salamandra* Linnaeus 1758.

Type locality: Monchique, Portugal (UTM 29 S 538349, 4130147).

Prevalence: 1/1 (100%) examined salamander was infected.

Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85541. No host was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360732).

Etymology: The specific epithet *steinhausi* is a genitive (possessive) Latin name (g. masculine). This patronym (eponym) honors Dr. Carl Otto Steinhaus (1870-1919), who was the first authority to describe a tetrasporocystic, dizoic oocyst in the Fire salamander.

Taxonomic remark: The only species of tetrasporocystic, dizoic coccidia species described from *S. salamandra* is *E. salamandrae* (Setinhaus 1889) Dobell 1909. The available information on *E. salamandrae* consists of an oocyst size of 18 x 30 µm. *Eimeria steinhausi* n. sp. (Table s3) presents an oocyst mean length of 21 x 27, longer and narrower 3 µm in mean respectively. Further morphological and molecular analyses shall be carried in future samples of *S. salamandra* to discard conspecificity and re-describe the former species. In addition, *E. tertia* Lavier 1936 described from the alpine newt, *Mesotriton alpestris* Laurenti 1768 from France presented an oocyst and a sporocyst that overlapped in size. However, *E. tertia* presented an OR which occupied half of the size of the oocyst and the sporocysts may present SB which lacks in *E. steinhausi* n. sp. or *E. salamandrae* (Duszynski *et al* 2007). Thus, the morphology and the host of *E. tertia* are fairly distinct from *E. steinhausi* n. sp. Until new molecular information were available on

the coccidia found in Caudata hosts we prefer to be conservative with the generic designation for this taxon. Considering the presence of SB in the former genus *Eimeria*, future studies concerning morphology, development characteristics and phylogenetic relationships on the coccidian species infecting Caudata hosts may be considered to classify them in a distinctly new genus.

***Combinatio nova* species**

***Choleoeimeria gallotiae* n. comb.** (Figure 1, photomicrograph B).

Synonym: *Eimeria gallotiae* Matuschka and Bannert 1987.
Type host: Canarian lizard, *Gallotia galloti* Oudart 1839 (Squamata: Lacertidae).
New locality for type host: La Balandra, Güímar, Tenerife (UTM 28 R 363151, 3126404).
Description: The sporulated oocyst (N= 7) is cylindroid with micropyle, PG and OR absent. The mean length is 29.4 µm ± 1.6 (SD), ranging 26.7-31.1 x 15.7 µm ± 0.4 (15.2-16.3) µm; OSI of 1.87 ± 0.1 (1.6-2.0). The oocyst is bilayered with an inner layer of approximately 0.5 µm, and an outer layer of approximately 0.6 µm. Micropyle, PG and OR are absent. No SB or SSB are observed in the sporulated sporocysts (N=7) which contained four ellipsoidal sporocysts which measure 12.2 ± 1.4 (10.5-14.4) µm x 7.3 ± 0.8 (6.6-8.9) µm; shape index 1.6 ± 0.1 (1.4-2.0). Each of the sporocysts presents two SP and a rounded SR which measured 5.8 ± 0.3 (5.5-6.4) µm x 5.1 ± 0.2 (4.8-5.4) µm.
Prevalence: 9/43 (20.9%) examined lizard were infected.
Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85542. No lizard was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360728).

Taxonomic remark

A previous tetrasporocystic, dizoic coccidia species was described in *G. galloti* from Tenerife. The exogenous oocyst of *E. gallotiae* (Matuschka & Bannert 1987) largely overlaps with the oocyst and sporocyst size, and the OSI of the coccidian species reported here (Table s4). Thus, we propose a combinatio nova species for the former *E. gallotiae*: i.e. *Choleoeimeria (E.) gallotiae* (Matuschka & Bannert 1987) n. comb. (from *Gallotia galloti* Oudart 1839, Tenerife, Islas Canarias, Spain).

***Acroeimeria* cf. *tarentolae* n. comb.** (Figure 1, photomicrograph G)

Synonym: *Eimeria tarentolae* Matuschka and Bannert 1986, in *Tarentola mauritanica* Linnaeus 1758 from Minorca, Balearic Islands, Spain.

New type host and locality: Tenerife wall gecko, *Tarentola delalandii* Duméril and Bribon 1836.

New locality for type host: Punta del Bocinegro, Guaza, Tenerife (UTM 28 R 332199, 3102128).

Description: The sporulated oocysts (N= 11) are oval 16.8 (mean) ± 1.1 (SD), range 14.9 to 18.3 µm x 12.7 ± 0.5 (11.8-13.5) µm; OSI of 1.32 ± 0.1 (1.1-1.5). The observed wall consisted of one single layer of approximately 1 µm thick. However, previous evidences suggest that the oocyst wall within Eimeriidae consist of two layers (Belli et al., 2006). Thus, the magnification employed to microphotograph this species could make difficult to distinguish more than a single layer. Micropyle, PG and OR are absent. The sporulated oocyst contains four ellipsoidal sporocysts (N= 9), 6.7 ± 0.4 (5.7-7.4) µm x 4.6 ± 0.4 (4.0-5.5) µm. SR absent, no SB or SSB are observed. There are two SP per sporocyst.

Sporulation: To the time of the observation of the samples, the oocysts were sporulated. The time of sporulation was not recorded.

Prevalence: 1/2 (50%) examined geckoes were infected.

Type material: Phototypes and DNA voucher are deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid under the accession number MNCN/ADN: 85543. No lizard was killed in the present study, so we could not deposit any symbiotype. The DNA sequence was deposited in the GenBank (KR360731).

Taxonomic remark

Eimeria tarentolae found in *T. mauritanica* from Minorca is the most similar species to *A.* cf. *tarentolae* n. comb. found in *T. delalandii* in this study. The size of both the oocyst and the sporocysts largely overlapped (see Table s5a, 5b, 5c and 5d). Nevertheless, molecular analyses of the species found in *T. mauritanica* from Minorca should be done for genetic comparison with the samples from *T. delalandii* from Tenerife to confirm conspecificity.

Other species

Based on the morphology and the phylogenetic position of *E. tropidura* closely related to *A. sceloporis* and *A.* cf. *tarentolae* n. comb. (Fig. 2), this species is also suggested to be

transferred into the genus *Acroeimeria*.

The proposed species is *Acroeimeria (E.) tropidura* (Aquino Shuster, Duszynski & Snell 1990) n. comb. (from *Tropidurus delanois* Baur 1890, Islote Osborn, Galápagos, Ecuador).

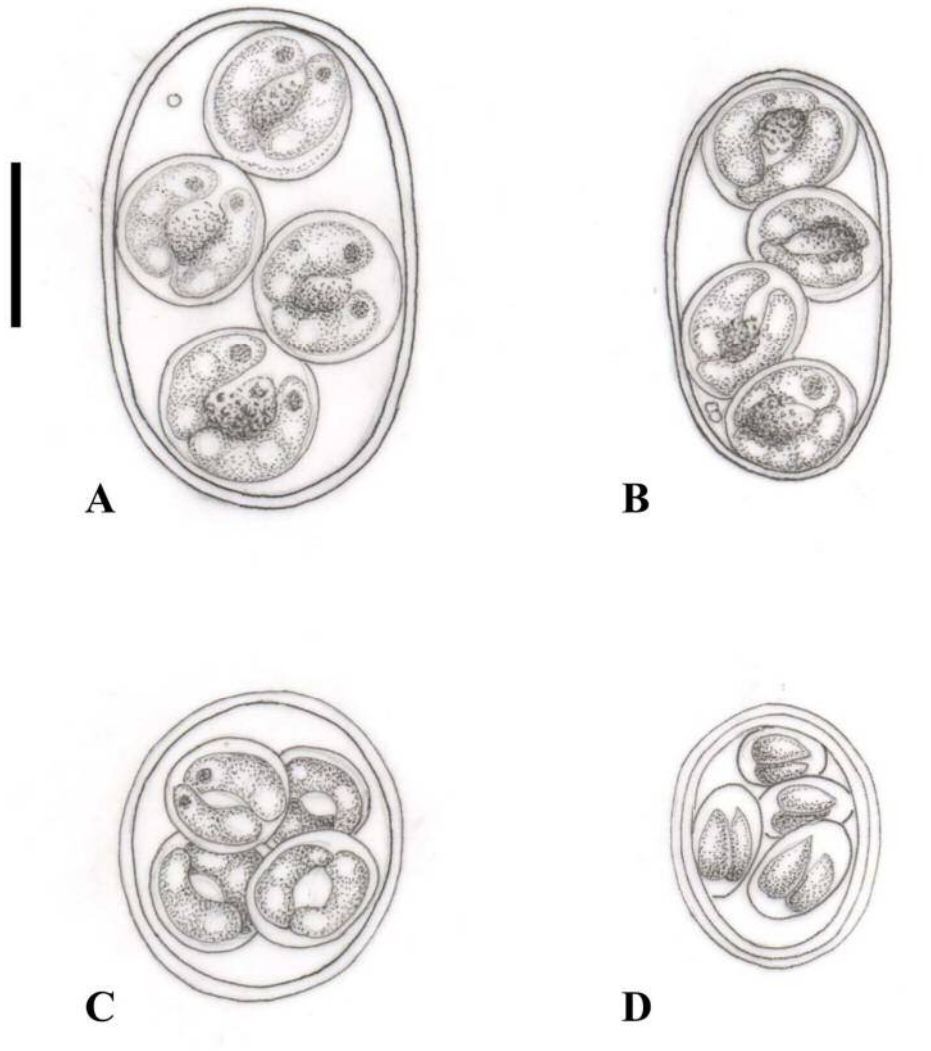

Figure s1. Line drawings of the newly described species of *Choleoeimeria* and *Eimeria* (i. s.) in this study. (A) *C. wiegmanniana* n. sp.; (B) *C. scincorum* n. sp.; (C) *E.* (i. s.) *eutropidis* n. sp.; (D) *E. steinhausi* n. sp. Scale bar = 10 µm.

Table s1. Species of tetrasporozoic, dizoic coccidia described in amphisbaenian lizards.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Choleoeimeria amphisbaenae	*Amphisbaena alba*	30-37 x 20-26 (33 x 22)	1.2-1.7 (1.5)	11-14 x 9-10 (13 x 9)	Lainson 2003
Eimeria witcheri	*Amphisbaena manni*	22-29 x 15-20 (26-18)	1.3-1.6 (1.4)	9-12 x 7-9 (10 x 8)	Huntington *et al* 1996
Eimeria amphisbaeniarum	*Amphisbaena manni*	26 -32 x 14-17 (29-15)	1.6-2.1 (1.9)	8-12 x 5-8 (10 x 6)	Huntington *et al* 1996
Choleoeimeria zarudnyi	*Diplometopon zarudnyi*	25-32 x 18-25 (27-22)	1.0-1.3 (1.2)	10-13 x 6-9 (11 x 7)	Abdel-Baki, Abdel-Haleem & Al-Quraishy 2013
Choleoeimeria wiegmanniana n. sp.	*Trogonophis wiegmanni*	26-31 x 18-20 (29-19)	1.3-1.6 (1.5)	8-10 x 7-9 (9 x 8)	This study

Table s2. Species of tetrasporocystic, dizoic coccidia of African and Asian Scincidae with available morphological information. *Molecular analyses or studies on the endogenous development of these species will confirm if they belong to the genus *Choleoeimeria*.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Choleoeimeria chalcides	*Chalcides ocellatus*	32-37 x 17-20 (35 x 19)	(1.88)	13-14 x 4.6-5 (14 x 5)	Probert, Roberts & Wilson 1988
Eimeria baltrocki	*Eumeces schneider*	35-45 x 17-20 (38 x 18)	*(2.01)	10-12 x 7-12 (8 x 11)	Daszak & Ball 1991
Eimeria (i. s.) *eutropidis* n. sp.	*Eutropis macularia*	13-15 x 12-15 (14 x 13)	0.9-1.1 (1.0)	5-8 x 4-6 (7 x 5)	This study
Eimeria pellopleuris	*Lygosoma pellopleurum*	28-35 x 12-15 (31 x 14)	*(2.2)	6-7 x 8-10 (7 x 9)	Bovee 1971
Eimeria auratae	*Mabuya* (s. l.) *aurata*	22-31 x 13-22 (28 x 18)	(1.5)	10-13 x 7-9 (12 x 8)	Al Yousif & Al-Rasheid 2001
Choleoeimeria scincorum n. sp.	*Mabuya* (s. l.) sp.	25-30 x 12-15 (27 x 14)	1.7-2.2 (1.9)	7-10 x 6-7 (9 x 7)	This study
Eimeria minetti	*Mabuya* (s. l.) sp.	18-21 x 12-14	(1.4)	(9 x 7)	Ray, Raghavarchari & Sapre 1942
Choleoeimeria jazanensis	*Scincus hemprichii*	25-27 x 14-16 (26 x 15)	(1.7)	10-12 x 6-8 (11 x 7)	Abdel-Baki, Al-Quraishy & Abdel-Haleem 2013
Choleoeimeria mitranusensis	*Scincus mitranus*	28-31 x 19-21 (29 x 20)	1.3-1.5 (1.4)	9-12 x 7-9 (11 x 8)	Al-Quraishy 2011
Eimeria scinci	*Scincus officinalis*	(36 x 25)	(1.4)	(14 x 10)	Phisalix 1923

Table s3. Species of tetrasporozoic, dizoic coccidia described in European and Asiatic caudata of the family Salamandridae. All the data were obtained from Duszynski, Bolek and Upton (2007).

Species	Host	Oocyst size range (mean)	OSI range (mean)	Sporocyst size range (mean)	Reference
Eimeria nipponensis	*Cynops pyrrhogaster*	44-55 x 31-38 (50 x 34)	(1.5)	-	Upton, McAllister & Trauth 1993
Eimeria pyrrhogaster	*Cynops pyrrhogaster*	38-45 x 34-45 (43 x 40)	(1.1)	(22 x 8)	Upton, McAllister & Trauth 1993
Eimeria saitamaensis	*Cynops pyrrhogaster*	23-26 x 23-26	(1.0)	(15 x 6)	Upton, McAllister & Trauth 1993
Eimeria spherica	*Mesotriton alpestris*	35 (22-38)	(1.0)	12-15 x 6-7	(Schneider 1887) Levine & Becker 1933
Eimeria tertia	*Mesotriton alpestris*	22-33 x 18-25 (26 x 21)	(1.2)	12-15 x 6-7	Lavier 1936
Eimeria canaliculata	*Triturus cristatus*	36-42 x 20-27 (39 x 24)	(1.6)	25-30 x 6	Lavier 1936
Eimeria propria	*Triturus cristatus*	38-41 x 22-24	(1.7)	18-22 x 7-8	(Schneider 1881) Doflein 1909
Eimeria grobbeni	*Salamandra atra*	10-1 x 9-10	-	5-6 x 4	Rudovsky 1925
Eimeria salamandrae	*Salamandra salamandra*	(30 x 18)	(1.6)	-	(Steinhaus 1889) Dobell 1909
Eimeria steinhausi n. sp.	*Salamandra salamandra*	25-28 x 21-22 (27 x 21)	1.1-1.3 (1.2)	12-13 x 8-9 (12 x 8)	This study

Table s4. Species of tetrasporozoic, dizoic coccidia described in lizards of the family Lacertidae. *Data from the redescription of the species in Al Nasr, I. S. (2011).

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
E. rountreei	*Takydromus tachydromoides*	31-39 x 24-32 (33 x 29)	(1.14)	13-17 x 10-13 (15 x 11)	Bovee 1971
E. takydromi	*T. tachydromoides, T. smaragdinus, T. sexlineatus*	28-27 x 21-17 (28 x 16)	(1.79)	8-11 x 8-7 (9 x 7)	Telford 1992
E. takydromi	*T. tachydromoides*	39-31 x 32-24 (33 x 29)	(1.07)	17-13 x 13-10 (15 x 11)	Telford 1992
**C. schmidti*	*Acanthodactylus schmidti*	31-39 x 24-32 (33 x 29)	(1.55)	11-14 x 8-10 (13 x 9)	Al Yousif, Al Sadoon & Al Shawa 1997
E. gallotiae	*Gallotia galloti*	29-33 x 14-18 (31 x 16)	(1.91)	12-17 x 8-11 (15 x 9)	Matuschka & Bannert 1987
Choleoeimeria gallotiae n. comb.	*Gallotia galloti*	27-31 x 15-16 (29 x 16)	1.6-2.0 (1.87)	10-14 x 7-9 (12 x 7)	This study

Table s5a. Species of tetrasporozoic, dizoic coccidia described in African geckoes. (*) information from Paperna and Landsberg, 1989; and (†) information from Ball and Daszak, 1995.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Eimeria tokayae	*Gekko gecko*	17-21 x 13-20 (18 x 18)	(1.01)	8-11 x 5-7 (9 x 6)	Ball & Daszak 1995
Eimeria tokayae	*Gekko gecko*	17-21 x 17-20 (19 x 19)	(1.06)	8-13 x 5-8 (10 x 7)	Present study
Eimeria bongaonensis	*Gekko gecko*	13-15 x 13-15 (14 x 14)	(1.0)	8-9 x 5-6 (9 x 5)	Sinha & Sinha 1978(†)
Eimeria simonkingi	*Gekko smithii*, *Gekko vittatus*, *Phelsuma lineata*	19-22 x 17-21 (20 x 19)	(1.06)	9-10 x 5-7 (10 x 6)	Ball & Daszak 1995
Eimeria vittati	*Gekko vittatus*	32-36 x 16-17 (34 x 17)	(2.03)	10-12 x 5-7 (11 x 6)	Ball & Daszak 1995
Eimeria helenae	*Hemidactylus brookei*	20-23 x 14-16 (22 x 15)	(1.4)	7-9 x 6-7 (8 x 7)	Bray 1984(*)
Eimeria scinci	*Hemidactylus flaviviridis*	(36 x 25)	(1.4)	(14 x 10)	Pellérdy 1974(*)
Eimeria furmani	*Hemidactylus frenatus*	18-24 x 14-19 (20 x 17)	(1.21)	9-10 x 6-8 (10 x 7)	Upton et al. 1990(†)
Eimeria rochalimai	*Hemidactylus mabouia*	28-31 x 15-18 (30 x 17)	(1.77)	10-12 x 7-9 (11 x 8)	Upton, Freed & Freed 1992(†)
Eimeria lineri	*Hemidactylus mabouia*	21-26 x 12-19 (24 x 16)	(1.53)	(10 x 8)	Paperna & Landsberg 1989

Table s5b. Species of tetrasporozoic, dizoic coccidia described in African geckoes. (#) Information from El-Toukhy et al., 2013; and (†) information from Ball and Daszak, 1995.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Eimeria lineri	*Hemidactylus turcicus*	25-28 x 18-21 (26 x 20)	(1.3)	9-11 x 7-8 (10 x 7)	El-Toukhy, Galal & Radwan 1997(#)
Eimeria pachybibroni	*Pachydactylus bibroni*	21-28 x 16-19 (26 x 18)	(1.44)	8-9 x 7-8 (9 x 8)	Upton, Freed & Burdick 1992(†)
Choleoeimeria pachydactyli	*Pachydactylus capensis*	25-31 x 11-17 (28 x 14)	(2.05)	10-13 x 6-7 (11 x 7)	Paperna and Landsberbg 1989
Eimeria rangei	*Pachydactylus rangei*	25-29 x 18-19 (27 x 19)	(1.43)	9-10 x 8-9 (10 x 8)	Upton, Freed & Burdick 1991(†)
Eimeria phelsumae	*Phelsuma madagascariensis grandis*	30-32 x 14-16 (32 x 15)	(2.12)	7-11 x 6-9 (10 x 7)	Daszak & Ball 1991(†)
Eimeria brygooi	*Phelsuma madagascariensis grandis, Phelsuma laticauda*	19-25 x 16-23 (23 x 21)	(1.1)	8-10 x 7-9 (9 x 8)	Upton & Barnard 1987(†)
Eimeria stebbinsi	*Phelsuma rosagularis*	16-19 x 11-13 (17 x 12)	(1.5)	7-8 x 3-6 (8 x 4)	Daszak, Ball, Jones, Streicker & Snow 2009(#)

Table s5c. Species of tetrasporozoic, dizoic coccidia described in African geckoes. (#) Information from El-Toukhy et al., 2013; and (†) information from Ball and Daszak, 1995.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Eimeria raleighi	*Phelsuma rosagularis*	16-19 x 14-17 (17 x 15)	(1.1)	7-8 x 6-7 (8 x 7)	Daszak, Ball, Jones, Streicker & Snow 2009(#)
Eimeria swinnertonae	*Phelsuma rosagularis*	21-25 x 17-18 (22 x 18)	(1.25)	8-10 x 6-8 (9 x 7)	Daszak, Ball, Jones, Streicker & Snow 2009(#)
Eimeria ptyodactyli	*Ptyodactylus hasselquistii*	21 x 24 (22)	(1.0)	10-11 x 8-9 (11 x 8)	Abdel-Aziz 2001(#)
Eimeria gizaensis	*Ptyodactylus hasselquistii*	29-30 x 22-24 (28 x 23)	(1.2)	9-10 x 7-9 (10 x 8)	Abdel-Aziz 2001(#)
Eimeria hailensis	*Ptyodactylus hasselquistii*	36-38 x 15-20 (38 x 17)	(2.2)	8-12 x 7-9 (10 x 8)	Abdel-Aziz 2001(#)
Eimeria barnardi	*Rhoptropus barnardi*	21-26 x 16-22 (24 x 20)	(1.22)	8-11 x 7-9 (9 x 8)	Upton, Freed & Burdick 1992(†)
Eimeria stenodactyli	*Stenodactylus elegans*	26-32 x 22-27 (28 x 24)	1.2	9-11 x 7-8 (10 x 8)	El-Toukhy 1994(#)

Table s5d. Species of tetrasporozoic, dizoic coccidia described in African geckoes. (#) Information from El-Toukhy et al., 2013.

Species	Host	Oocyst size Range (mean)	OSI Range (mean)	Sporocyst size Range (mean)	Reference
Eimeria alexandriensis	*Tarentola mauritanica*	23-30 x 14-19 (26 x 17)	(1.6)	10-17 x 6-8 (13 x 7)	El-Toukhy, Abdel-Aziz, Abo-Senna & Abou El-Nour 2013
Eimeria tarentolae	*Tarentola mauritanica*	18-19 x 13-14 (18 x 13)	(1.3)	6-7 x 6-7 (7 x 7)	Matuschka & Bannert 1986
Acroeimeria tarentolae n. comb.	*Tarentola delalandii*	15-18 x 12-13 (17 x 13)	(1.32)	6-7 x 4-5 (7 x 5)	Present study
Eimeria delalandii	*Tarentola delalandii*	42-48 x 20-26 (45 x 22)	(2.04)	12-15 x 9-11 (14 x 10)	Matuschka & Bannert 1986
Eimeria dahabensis	*Tropiocolotes nattereri*	24-33 x 18-24 (29 x 21)	(1.38)	14-17 x 7-10 (15 x 9)	Abou El-Nour 2005(#)
Eimeria tripolitani	*Tropiocolotes tripolitanus*	20-28 x 17-19 (25 x 18)	(1.38)	7-10 x 7-9 (9 x 8)	Abdel-Aziz 2001(#)

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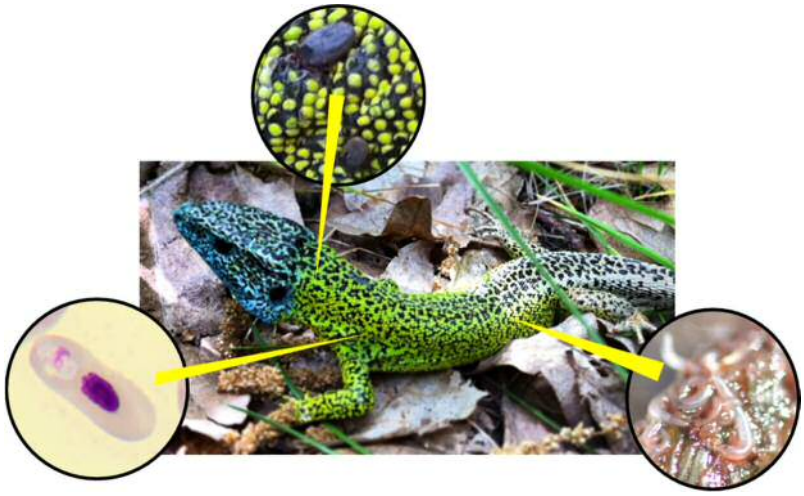
CHAPTER II



Signaling the individual quality in lizards: Colours and parasites in different host-parasite systems

In this chapter three correlational studies in three different host-parasite systems suggest that visual ornaments in lizards may be influenced by different parasitic infections in different host-parasite systems. However, the peculiarities associated with the physiology of the pigments may be important to interpret the differences found between infected or not infected individuals, or individuals with high intensities of infection and low infected host individuals. In addition, the results of two of these studies that compared ornaments in males and females of the same population suggested that selection "awards" chromatic dimorphism in contexts of high incidence of parasitosis.

MELANIN AND CAROTENOIDS ALLOCATION TO COLOUR ORNAMENTS OF *LACERTA SCHREIBERI* REFLECTS DIFFERENT PARASITIC DISEASES

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Abstract

Host species in populations under high pressure imposed by parasitic diseases may evolve ornaments to signal the individual host quality to conspecifics. The oxidant handicap hypothesis predicts that a trade-off in the redistribution of carotenoids in individuals subjected to oxidative stress will occur between the skin and the antioxidant machinery. In addition, high oxidant conditions may favour eumelanogenesis and thus, the conspicuity of eumelanin-based traits in lizards. Thus, visual ornaments may be more conspicuous in individuals with the best genetic quality to cope with oxidative stress imposed by parasites. In this study, we investigated the effect of three types of parasites (hemoparasites, ixodid ticks and intestinal nematodes) over the conspicuousness of the throat blue and yellow patches in males of *Lacerta schreiberi*. The individuals infected by hemoparasites of the genus *Schellackia* showed throat blue patches with the greatest levels of UV to blue chroma. Similarly, lizards infected with *Schellackia* tended to have fewer values of hue in the yellow patch. Furthermore, the number of attached ticks and the body condition negatively correlated with the brightness in the throat yellow patch in compliance with the Hamilton and Zuk's hypothesis. All these results suggested that lizards can convey the cost caused by their parasitic diseases through their throat coloured patches. In addition, we explored the spectral variation from lizard skin by either removing the carotenoids or oxidizing the melanin present in the integument. These experiments altered the reflective properties from the patches and thus, in line with the oxidant handicap hypothesis, we propose that a balance between both the carotenoid and the melanin reallocation during an oxidant challenge accounts for the total reflectiveness in both carotenoid- and structural-based coloured patches. These results may explain why brightness is a predictor of individual quality in many lizard species.

Keywords: Hamilton & Zuk, handicap, Lacertidae, parasite, reptile, visual communication

Introduction

The handicap hypothesis (Zahavi, 1975) proposed sex modelling honest traits in populations with selective pressures that compromise the integrity of the secondary sexual characters. Parasitic diseases are one of the main selective pressures in nature inducing selection for good quality genes (Hamilton and Zuk, 1982). Individuals of the eligible sex from a population subjected to a high pressure by parasitic diseases may be able to express their genetic quality in terms of resistance or tolerance to those infections through several types of signals directed to different sensorial channels (Martín et al., 2007 and 2008).

Visual ornaments based on either pigments or structures present in the skin of lizards serve as honest signals acting as visual cues of individual quality to conspecifics (Hews, 2006; Calisi et al., 2008; Bajer et al., 2010, 2011; Molnár et al., 2013). Therefore, colourful traits based on pigments (carotenoids and melanin) might be honestly mirroring the individual ability to cope with the physiological trade-offs underwent by the bearer. In this sense, the oxidation handicap hypothesis (OHH; Alonso-Álvarez et al., 2007) predicts a trade-off in the allocation of antioxidant molecules, such as carotenoids, during an oxidative challenge. Thus, the organisms may allocate the carotenoids obtained in the diet into the antioxidant machinery, or rather into the chromatophores, and so, varying the showiness of the sexual ornamentation (Cote et al., 2010).

The typical organization of the dermis of lizards from the basal layer to the dermal surface may include a layer of conjunctive tissue that may reflect in the full range of wavelength (Jacot et al., 2010; Olsson et al., 2013); typically, over this basal layer there is a layer of melanophores that harbour melanin, responsible of brown and black colours; then, one or several layers of crystals of guanine structured in platelets, responsible of UV-blue colouration (Pérez i de Lanuza and Font, 2010); and an outer layer of cromatophores that can contain a combination of pteridines and carotenoids (Steffen and McGraw, 2007; Olsson et al., 2013). Thus, visual ornaments in lizards are the result of the combined effect of spectral reflectance from these layers of structures and pigments that are located in the dermis (Grether et al., 2004; Kuriyama et al., 2006; Saenko et al., 2013). For instance, experimentally-induced deposition of melanin in the melanophores of the skin of lizards resulted in an enhancement of the reflection from the structures in the above layers (Quinn and Hews, 2003; Cox et al., 2008). Otherwise, carotenoids chemically removed from the first layers of the skin of lizards revealed the underlying structural colour (Fitze et al., 2009; Saenko et al., 2013). Likewise, in other study it was suggested that the presence of ~25% of blue-bellied males of *Iberolacerta martinezricai* in a typically green-bellied population might be consequence of the absence of yellow pigmentation in the skin of these individuals (Arribas, 2008).

Parasites are known to produce cellular damage and in consequence, oxidative stress on their hosts (e. g. Atamna and Ginsburg, 1997; Mougeot et al., 2009). Furthermore, the

immunocompetence-handicap hypothesis (IHH; Folstad and Karter, 1992) predicts an increase of testosterone levels prior to the breeding season in the eligible sex which may be costly to the organism compromising the immune response (Belliure et al., 2004; Oppliger et al., 2004). Therefore, a combined effect of androgenic hormone levels and infection stress may impose a high oxidative imbalance to organisms during the breeding season (Salvador et al., 1996; Salvador et al., 1997; Veiga et al., 1998; Mougeot et al., 2009). In natural populations it is common to find several parasites infecting the same individual during the breeding season and the handicap that different parasitosis may impose to the physiology of melanin- and carotenoid-based ornaments is to date barely studied (i.e. McGraw and Hill, 2000; Fitze and Richner, 2002).

The Schreiber's green lizard, *Lacerta schreiberi* (Squamata: Lacertidae) is one of the most colourful lizards in the Iberian Peninsula. The individuals of both sexes present a shiny green back, a bright yellow throat and a belly with black dots. In addition, the males during the breeding season present a bright blue head and throat (Figure 1a). These colour patches in *L. schreiberi* are more conspicuous to conspecifics than to predators suggesting a role as intraspecific visual signals (Pérez i de Lanuza and Font, 2014), and also show correlative relations with physiological and behavioral variables (Martín and López, 2009). Specifically, the dominance status of the males was negatively related with the brightness and positively related with the UV-blue chroma both from the throat blue patch. In addition, the throat UV-blue chroma and the yellow chroma from the chest were negatively correlated with the inflammatory response of the skin to an immune challenge, suggesting a trade-off in the allocation of antioxidants between the chromatophores of the skin and the antioxidant machinery during the challenge (Alonso-Álvarez et al., 2007; López et al., 2009). However, the yellow patch from the chest was not correlated with the dominance status of the male lizards, although paired males found guarding females in the field showed more saturated yellow chests. This suggests a differential role for the colourful patches between intra- and intersexual communication (Martín and López, 2009).

In this study, we investigated whether either the structural or the pigment-based throat ornamentation of the male Schreiber's Green lizards may reflect the parasitic diseases co-occurring in the population. We expected that individuals with lower infections showed more showy ornaments than lizards severely infected (Hamilton and Zuk, 1982). Furthermore, we studied the variation in the spectral properties of the skin of the lizards by removing the carotenoids and oxidizing the melanin present in biopsied skin from the throat of three freshly dead lizards under experimental conditions. Integrating both the experimental results in the lab and the correlational data in vivo, we discuss about the constraints that parasitic diseases may impose in the redistribution of pigments in the skin of *L. schreiberi*.

Material and Methods

Sampling lizards and parasites

During the mating season of 2012, 21 adult males of *Lacerta schreiberi* were collected with a noose in a deciduous forest in Segovia, Spain (40.88814,-4.02827). Each lizard was measured to the nearest millimeter with a ruler and weighted to the nearest decigram with a digital balance. The body condition of the individuals was estimated with an index (BCI) calculated as the residuals of the correlation between the snout-vent length (SVL) and the weight (see Schall and Pearson, 2000 but also Green, 2001). The individual age was estimated as the number of arrested lines (LAGs) in one phalanx obtained from each lizard using common techniques in skeletochronology (see below). To remove the effect of the age over body condition index we included, as a cofactor, the number of LAGs found in the phalanxes of the individuals in the correlation and we used the residuals of this analysis as the new variable of BCI. In addition, we counted the number of attached ticks to the lizards and we recover fecal and blood samples from the animals for studying the presence and absence of intestinal nematodes and blood protozoa respectively. With this purpose, we made thin layer blood smears from each lizard to survey for hemoparasites of the genus *Schellackia* what is the main parasite found in the blood in this population (see Megía-Palma et al., 2013). Smears were immediately air-dried and then fixed with methanol (Rogier and Landau, 1975) and stained during 40 minutes with Giemsa diluted 1:10 in buffer, pH 7.2 (Schall, 1986). We screened 15.000 red cells in each blood smear in search for infected blood cells to assign each lizard into the category of infected or uninfected. In a previous study in this population we got a 100% correlation between molecular and microscopic prevalence of hemococcidia (Megía-Palma et al., 2013). We also screened fecal samples for intestinal nematodes, which are prevalent in this lizard species in the Sistema Central mountains (Roca et al., 1990). Fecal samples obtained from each individual by briefly massaging their belly were stored at 4 ºC in 1.5 mL vials (Eppendorf Tubes® 3810X, Eppendorf Iberica, Madrid, Spain). Nematode eggs were concentrated by means of Sheather's sugar flotation technique (Levine, 1973; Dryden et al., 2005), and then we screened the samples for nematodes at 200X magnification.

Aging the lizards

Aging individuals by skeletochronology was proven to be a valid technique in the Schreiber's green lizard (i. e. Luís et al., 2003). Thus, to know the age of each animal in this study the second toe of the left hind limb of each lizard was removed using common techniques of toe-clipping which are innocuous to lizards (i.e. Perry et al., 2011). We removed the toe with surgical scissors that were sterilized with ethanol. Then, the limb affected by removal of the toe was treated with liquid plaster to avoid infections (Sprayed Plaster, Hansaplast, Beiersdorf, Hamburg, Germany).

The clipped toes were stored at 4ºC in 10% formaline prior to process them. Then, the piled phalanx were decalcified in 4% nitric acid during 3-5 minutes and then stained with Ehrlich's hematoxylin. The stained phalanxes were cut to 12 µm thick slides with a microtome (Microm HM-505N Cryostat Microtome). The resulting slices (Figure 1) were re-stained with Ehrlich's hematoxylin to improve the visibility of the lines of arrested growth (LAGs; sensu Castanet, 1975). Microscope slides with 10-15 slices from each individual were prepared with samples from two phalanxes of the same toe. The same person assigned a number of LAGs to each sample by observing the preparations at 400X and choosing the most repeated observed number of LAGs per slide.

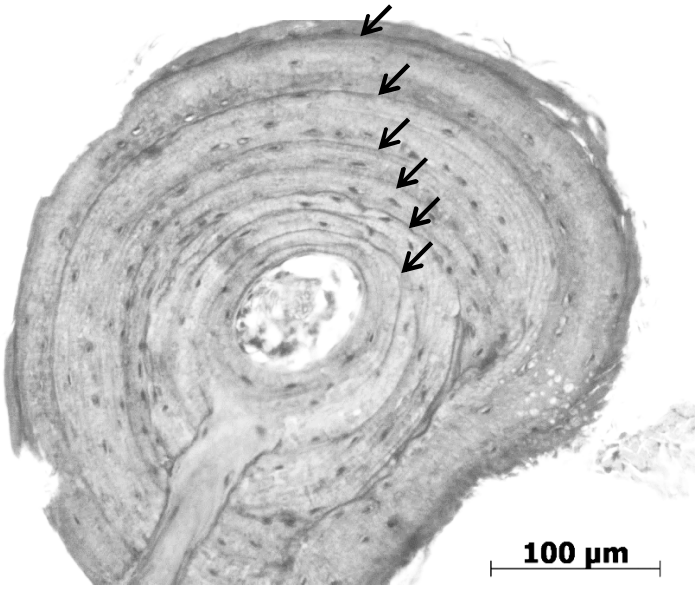

Figure 1. Microcut of one phalanx of *L. schreiberi* with six lines of arrested growth. Picture taken by Carolina García-Garrido.

Measurement of the throat blue and yellow patches in the field

With the aid of a spectrophotometer (Jaz DPU® Module) we measured in the field three consecutive times the reflectance spectrum from 320 to 700 nm in a central area from both the blue and the yellow throat patches of 21 adult Schreiber's green lizard males (Figure 2a). The three measurements were tested for repeatability (>74%) and they were averaged for further calculations. The spectrophotometer used one Pulsed Xenon Light Source (Jaz-PX) connected to an optical fiber. The probe was mounted within a holder that ensured readings were taken from areas 1 mm in diameter at a constant distance of 3 mm from the skin surface with a 90º angle (Endler, 1990; Martín and López, 2009; Bajer et al., 2010; Pérez i de Lanuza and Font, 2010). All the measurements were relative to a 99% WS-1 white reflectance standard (all the components from Ocean Optics Inc., Dunedin, FL, USA).

Measurement of the throat blue and yellow patches in the lab

We obtained skin samples from the skin of three freshly dead male lizards recovered in the area of study that were frozen immediately at -20 ºC. In the lab, we prepared two sets of six biopsied skin strips from the throat of the dead lizards. Three of the strips of each set were cut from the blue patch and the remaining three from the yellow patch of the three dead lizards respectively. We fixed the biopsied skin on a flat surface to be able to do repeated measurements in the same area of the strip as we did in the field with living lizards. The carotenoids were removed from three blue and three yellow strips by including the biopsied skin in a bath of acetone (100% p/v; AnalaR NORMAPUR). The acetone dilutes differentially carotenoids from other pigments present in the tissue (Saenko et al., 2013). During the first two hours we quantified the spectrum from the biopsies every 10 minutes following the same methods described above. Then, the biopsied skin was left 12 hours more in acetone and a final measurement was done. A second set of six biopsied skin strips were submerged in a bath of hydrogen peroxide (33% p/v; Panreac Química S.A.U.) repeating the spectral measurements every ten minutes during two hours. The hydrogen peroxide oxidizes the melanin (Napolitano et al., 2000), altering the reflective properties of the biopsied skin. Then the variables for brightness, chroma and hue were calculated following the same methods used for measurements obtained from lizards in the field. Non-parametric Friedman ANOVAs for multiple dependent samples were performed between the consecutive measurements to explore the variation in the skin spectral properties when we decreased the concentration of carotenoids or oxidized the melanin in the skin samples.

Statistical analyses of colour ornaments

We analyzed the spectral data from both the blue and the yellow throat patches from the lizards and the biopsied skin adapting the segment classification method for spectral analysis (Endler, 1990; Grill and Rush, 2000). Thus, we selected the spectral segments to explore as follows. Considering the morphology of the spectrum from the blue patch with two peaks in the near UV-blue (Figure 2b), we divided the spectrum from 320 to 475 nm (UV-blue spectrum) for short wavelengths and 475 to 700 nm for mid to long wavelengths. In relation to the yellow patch it showed a single peak in the UV region and a second peak in the yellow region (Figure 2c). Thus, we considered the region from 320 to 400 nm for short wavelengths and from 450 to 700 nm for mid to long wavelengths (i.e. carotenoid chroma; Montgomery, 2005). With this classification of the spectral data, we calculated the relative chroma for each specific segment defined above as $\Sigma Q_{segment}/\Sigma Q_T$ where Q is the value of reflectance for each considered wavelength. In a preliminary analysis of the blue patch we found a high negative correlation between the relative chroma in the range 320 to 475 nm and the range from 475 to 700 nm (r^2= 0.99; p= 0.0000). Therefore, we calculated the relation between short and long wavelengths dividing the relative chroma in the

UV-blue region between the relative chroma in the yellow-red region and using this as a value of chroma in further analyses. Furthermore, the total brightness for each spectrum was calculated as ΣQ_T, whereas the hue was calculated as the value of wavelength (λ) for the Q_{max} (i.e. λmax; Montgomery, 2005).

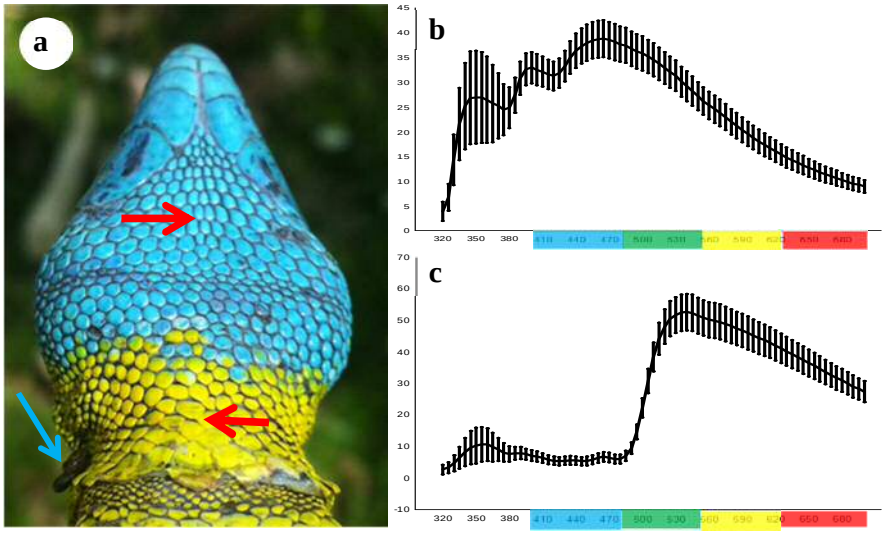

Figure 2. (**a**) Red arrows: blue and yellow patches in the throat of males Lacerta schreiberi. Blue arrow: attached ixodid ticks next to the collar. Spectral data (mean ± standard error) from the blue (**b**) and the yellow (**c**) throat patches.

To test the effect of the co-occurrence of parasitic diseases over the spectral properties of the throat in the males of the population we performed GLM models in Statistica 10.0 (Statsoft Inc.) and the residuals of these models were checked for normality and homocedasticity. The spectral variables: i.e. brightness, chroma and hue, were the dependent variables and date of capture, BCI_AGE, number of ticks per lizard, the status of infection by both blood parasites and nematodes were the independent variables in the models.

Ethical note

The lizards included in this study were captured using a noose (a pole with a loop of string with a slipknot that tightens around the neck of the lizard). In the same spot where the lizards were captured in the field, we performed the spectrophotometric measures of the two throat patches in a shady place avoiding the stress of transporting them. The toe used for aging the lizards was clipped with sharp surgical scissors that were previously sterilized with ethanol. This method is similar to toe-clipping which is commonly used to marking lizards and was evidenced to be the most innocuous marking technique (i.e. Langkilde and Shine, 2006; Perry et al., 2011). Then, the limb affected by removal of the toe was treated with liquid bandage to avoid infections (CURAD® Spray Bandage, Beiersdorf, Hamburg, Germany).

In relation with the blood samples for detecting hemoparasites, after cleaning the base of the tail with ethanol we obtained two drops of blood using a sterile needle. We made the incision always at least 3 cm away from the cloaca to avoid the hemipenes. We used 75 µL microcapilars, and the amount of blood taken was fewer than the 10% of this volume. One study evaluated the stress induced by sampling 70 µL of blood from a lizard (Langkilde and Shine, 2006), in spite of that amount the method resulted in low stress levels for the individuals (Langkilde and Shine, 2006). Moreover, in comparison to other methods which get the blood from the postorbital sinus, the technique used here seems innocuous to lizards. The wound was occluded until it stopped bleeding and the area was cleaned with ethanol. In addition, approximately three fourths of the lizards dropped fecal boli at the moment of their capture. The remaining individuals were stimulated by briefly rubbing their venter (e.g. Herrel et al., 2006). All the lizards were released after approximately 15 minutes of handling and they behaved normally running to hide.

In this study we included an experimental section where we chemically treated skin strips from voucher individuals found in the field site. Apparently, the deaths of these three individuals had different origin. The first male was found run over in the road that crosses the area of study. The second one was found with severe wounds that had caused its death. Feral cats are observed close to the area. Is common that these animals play with the lizards causing their death, and after killing them they leave the corps. The third individual had participated in the study and was found dead several days after its manipulation close to the location where it had been first captured. The corps did not present any apparent injury and we can not discard that its death was precipitated by the stress of handling (Moore et al., 1991). All the corpses were in good conditions and were used to obtain novel and valuable information (Rollin and Kessel, 1998) to the study of the reflectivity properties of lizard skin.

Results

Field data correlations

The mean weight ± SE and range for the lizards was 31.0 ± 4.7, 23.9-40.4 g. The mean SVL ± standard error (SE) and range of the lizards was 102.5 ± 5.2, 93-111 mm. Thus, all the lizards included in the analyses exceeded the minimum adult SVL described for the Schreiber's Green lizard in the Sistema Central (Galán, 1984). Furthermore, all the males had the characteristic blue head that is typical during the mating season in the adult males of this species. Indeed, the number of LAGs in the phalanx was ≥ 4 (4 to 7), which agrees with the age of sexual maturity of the species (Marco, 1995). The 95% (20/21) of the individuals studied here showed ticks (*Ixodes ricinus*) during the period of study that were attached to the shoulders, the neck and the tympani of the lizards (Figure 1a; blue arrow). The mean number of ticks ± standard error (SE) and range was 19.8 ± 15.2 (0-46). Twelve of the 21 lizards sampled (57%) were infected by *Schellackia* sp.

parasites and 38.0% of the lizards were infected by intestinal nematodes for which only prevalence was recorded.

The brightness in the throat yellow patch was significantly and negatively related to the tick load ($F(1, 15)= 5.08$; $p= 0.03$; Figure 3a). Furthermore, this trait was dependent of BCI_AGE ($F(1, 15)= 5.04$; $p= 0.04$; Figure 3b) suggesting that is a condition dependent signal costly to maintain (Zahavi, 1975). Furthermore, the individuals infected by *Schellackia* tended to have fewer values of hue ($F (1, 15)= 3.6$; $p= 0.07$; Figure 3c). In relation with the throat blue patch those males infected with *Schellackia* showed higher levels of chroma ($F (1, 14)= 7.6$; $p= 0.01$; Figure 3d). We did not find any significant relation between intestinal nematode prevalence and patch colour (data not shown).

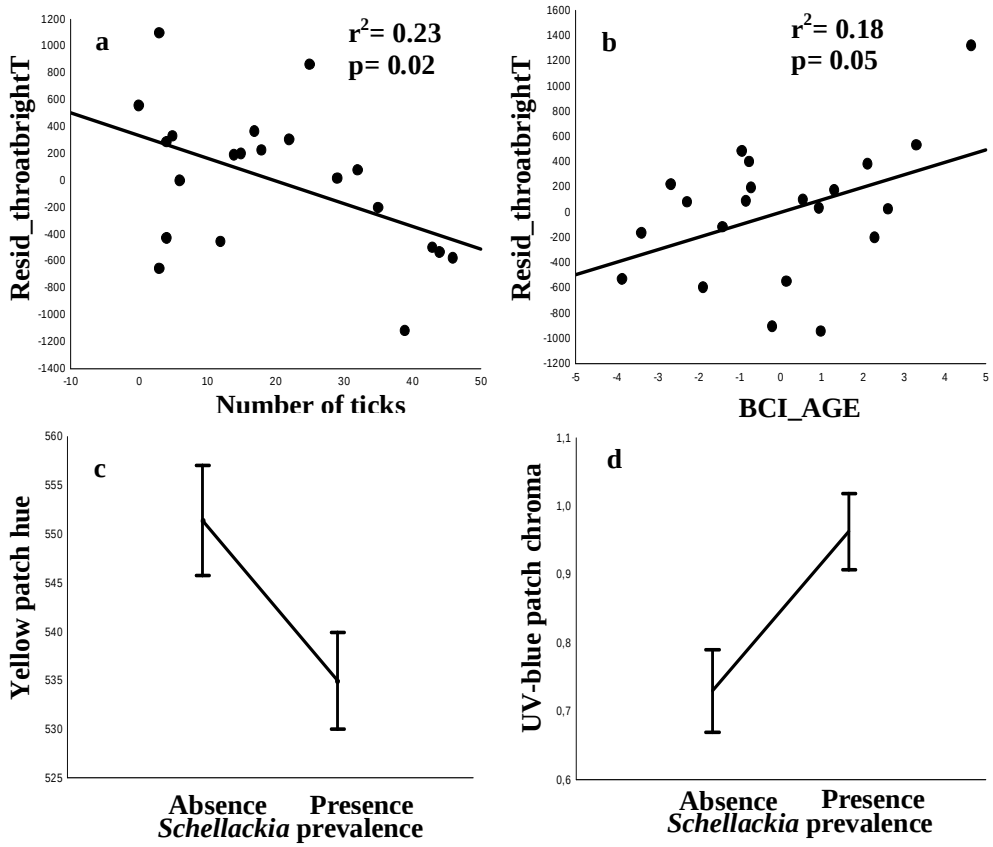

Figure 3. (**a**) Residuals of the regression of the throat yellow patch brightness with the variables date of capture, nematode presence, *Schellackia* presence and BCI correlated to the number of ixodid ticks attached to the skin, and (**b**) relation of the residuals of the regression of the throat yellow patch brightness with the variables date of capture, nematode presence, *Schellackia* presence and tick load with the body condition index corrected by age. (**c**) GLM plot (mean ± standard error) of the yellow patch hue controlled by presence of *Schellackia* parasites. (**d**) GLM plot (mean ± standard error) of the throat blue patch chroma controlled by presence of *Schellackia* parasites.

Carotenoids extraction and melanin oxidation

The oxidization of the biopsied skin from the throat yellow patch (Figure 4a) provoked that the brightness and the UV-blue chroma tended to increase (Friedman ANOVA brightness: χ^2= 3.0; p= 0.08; $chroma_{UV\text{-}blue}$: χ^2= 3.0; p= 0.08). Whereas the carotenoid chroma tended to decrease (Friedman ANOVA $chroma_{carotenoid}$: χ^2= 3.0; p= 0.08). The hue did not significantly change during all the treatment (Friedman ANOVA hue: χ^2= 9.1; p= 0.33).

Removing the carotenoids with acetone in the throat yellow patch (Figure 4b) significantly increase the brightness after 100 minutes (Friedman ANOVA: χ^2= 19.5; p= 0.03). The $chroma_{320\text{-}400}$ from the yellow patch tended to increase after 120 minutes of treatment with acetone (Friedman ANOVA: χ^2= 17.3; p= 0.09), whereas the $chroma_{450\text{-}700}$ in this patch significantly decreased after 100 minutes of treatment (Friedman ANOVA: χ^2= 20.7; p= 0.02). The hue of the yellow patch tended to change after 80 minutes of treatment with acetone (Friedman ANOVA: χ^2= 13.5; p= 0.09).

Similarly, the experimental oxidization of the melanin in the biopsied skin from the blue patch (Figure 4c) revealed a significant increase in the brightness after 60 minutes of treatment (Friedman ANOVA χ^2= 12.8; p= 0.04). Whereas the experimental extraction of carotenoids from the biopsied skin of the blue patch (Figure 4d) provoked a nearly significant increase of the total brightness of this patch after 120 minutes of treatment (Friedman ANOVA: χ^2= 19.3; p= 0.05). The acetone treatment did not statistically affect the chroma from the blue patch during the first two hours of the experiment. However, the oxidization of the biopsied skin from this blue patch modified the relative contribution of short and long wavelengths to the total spectrum. The relative chroma from 320 to 475 decreased after 120 minutes (χ^2= 16.9; p< 0.05) and the relative chroma from 475 to 700 tended to increase after 60 minutes (χ^2= 10.0; p= 0.07). The hue in this blue patch did not change during the oxidization (Friedman ANOVA: χ^2= 3.0; p= 0.93), or even after the overnight treatment with acetone (Friedman ANOVA: χ^2= 8.7; p= 0.72) since the values of λmax remained stable in the UV region (Figures 4c and 4d).

Discussion

Our results suggest that a balance in the allocation of melanin and carotenoids in the throat coloured patches in the males of L. schreiberi can mirror different parasitic diseases. Specifically, lizards with more attached ticks showed a duller throat yellow patch and worse body condition in compliance with the Hamilton and Zuk's hypothesis (1982). During the mating season the lizards of this population presented *I. ricinus* attached to the skin which practically disappeared at the end of this period (Pers. Obs., R. M.). This fact may be mirroring an increase in testosterone levels during the mating season (Folstad and Karter, 1992) since this hormone increases the susceptibility of lacertids to be infested by ticks (Salvador et al., 1996; Olsson et al., 2000). In our

study, the chroma and the hue were not correlated with the tick load in the throat yellow patch, thus the decrease in brightness might be related to other pigments, such as melanin rather than carotenoids. Indeed, the testosterone induces the deposition of melanin in the basal layers of the skin of lizards (Quinn and Hews, 2003; Cox et al., 2008) decreasing the total brightness from the patch and enhancing the relative chroma in short wavelengths (Cox et al., 2008). Consistently, when we experimentally oxidize the melanin in the biopsied skin from the yellow patch the brightness rapidly increased. The absorbance of wavelengths within the blue range in the visible part of the spectrum after the treatment (Figure 4a) suggested the persistence of the carotenoids in the skin (see Jacot et al., 2010). The increase in testosterone levels during the mating season may impose a handicap to the individuals increasing the oxidative stress and the susceptibility to parasites (Folstad and Karter, 1992; Salvador et al., 1996). Since ticks may reduce the levels of circulating testosterone in small vertebrates (Müller et al., 2013), the yellow patch may signal the ability of the males to cope with tick infection by maintaining optimal testosterone levels needed for signalizing and reproductive functions. This is supported by the positive relation between brightness and body condition in this patch (Figure 3b). Furthermore, lizards with chronic infections by *Schellackia* tended to have lower values of hue in the throat yellow patch than uninfected individuals. Although this result should be taken cautiously because the difference was not significant, it suggests a slight effect of the parasite on the hue of this patch. In this sense, as (i) changes in the guanine platelets aggregation in the iridophores were proven to account for hue changes in carotenoid-based traits in the common lizard (San Jose et al., 2013), (ii) platelet distance can be induced by neural and hormonal changes (see Teyssier et al., 2015) and (iii) chronic levels of an adrenal stressor negatively affected the hue of a carotenoid-based trait in the common lizard (Fitze et al., 2009), we can hypothesize that part of the variation of hue parameter in the throat yellow patch of *L. schreiberi* might be the consequence of changes in the platelet aggregation mediated by chronic stress related to infection by *Schellackia*.

Former studies exploring the relation between inter-individual variance in colouration in green lizards and infection status are based on infections by endoparasites of the genera *Hepatozoon* (Apicomplexa: Adeleorina) and *Karyolysus* (Apicomplexa: Adeleorina), whereas in the present study the blood parasites belonged to the genus *Schellackia* (Apicomplexa: Coccidiasina). All these parasite genera are responsible of chronic infections in vertebrate hosts and are transmitted when the lizard swallows an infected blood-sucking mite of the genus *Ophionyssus* (Reichenow, 1920; Haklová-Kočíková et al., 2014).

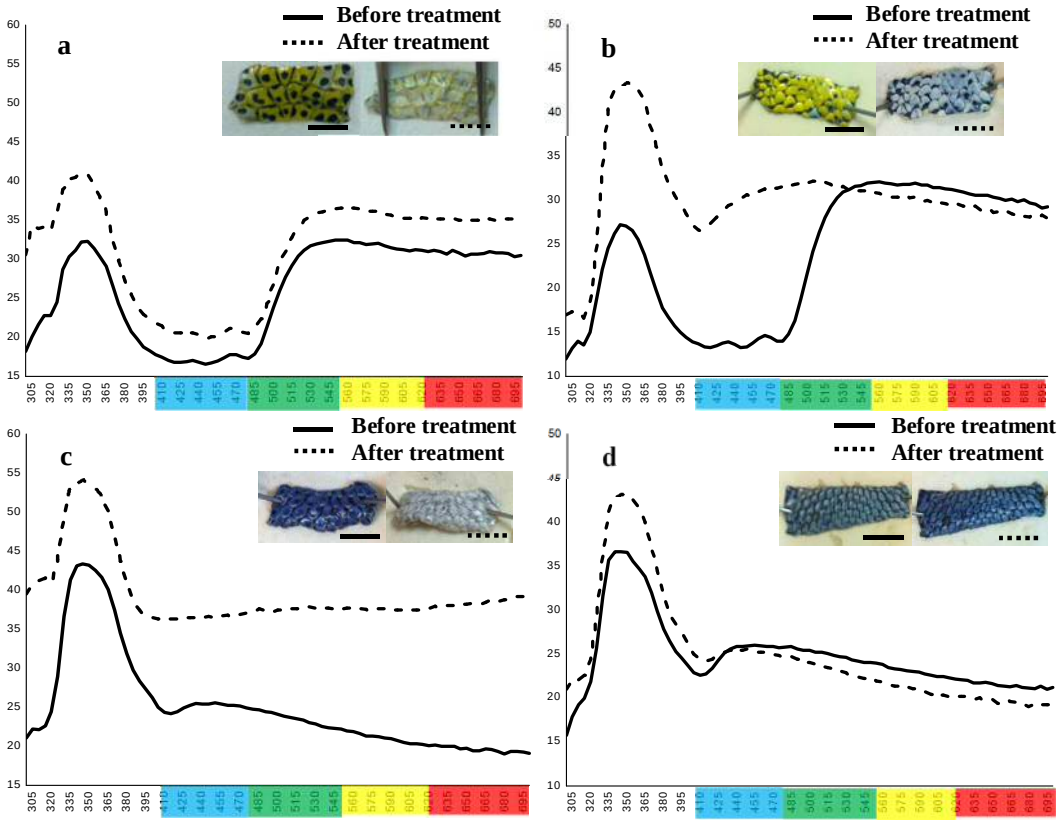

Figure 4. Spectral curves showing the averaged reflection of the biopsied skin from the throat yellow patch treated with hydrogen peroxide (**a**) and acetone (**b**); and the same treatments: hydrogen peroxide (**c**) and acetone (**d**) applied to biopsied skin from the throat blue patch.

However, the life cycles of these blood parasites fairly diverge. *Karyolysus* and *Hepatozoon* undergo their development in the host liver cells, where they cause tissue damage (Telford, 2008). However, parasites of the genus *Schellackia* undergo their development in the gut walls where they destroy the cells after several cycles of merogony which is characteristic of these hemococcidia (Telford, 2008). The successive cycles of wall ruptures in the gut cells might provoke a reduction in the assimilation of nutrients (Hõrak et al., 2004). Since the carotenoids can only be incorporated into the organism through the diet (Schantz et al., 1999), a reduction in the nutrient absorption in the gut wall might reduce the assimilation of carotenoids. Therefore, those individuals chronically infected by *Schellackia* might suffer a trade-off in the reallocation of the available carotenoids in the organism that are invested to regulate the oxidative balance (Galván and Solano, 2008; Mougeot et al., 2009; Sepp et al., 2012).

In relation with the throat blue patch, the lizards infected by *Schellackia* showed higher levels of UV-blue chroma but fewer $chroma_{475\text{-}700}$ in this area. This could be due to the increase in testosterone levels during the mating period (Folstad and Karter, 1992). The nuptial testosterone levels along with the cellular damage provoked by chronic infections of parasites may increase

the oxidative imbalance (Sepp et al., 2012). The synthesis of eumelanin, which is the main type of melanin occurring in the melanophores from the skin of lizards (but see Roulin et al., 2013), is favoured under depleted levels of reduced glutathione (GSH) (Galván and Alonso-Álvarez, 2008) which leads to high oxidant conditions (Galván and Solano, 2008, 2009). Oxidative stress may be mediated by parasitic diseases (Atamna and Ginsburg, 1997; López-Arrabé et al., 2015), or high levels of testosterone (Alonso-Álvarez et al., 2007). This is congruent with experimental results in phrynosomatid lizards which revealed that increased levels of testosterone resulted in a pleiotropic deposition of melanin in the dermal basal layers (Quinn and Hews, 2003). This deposition of melanin significantly decreased the brightness, and increased the chroma in two different blue patches (Cox et al., 2008). Likewise, lizards chronically infected with malaria parasites were darker than the healthy ones (Ressel and Schall, 1989), whereas common lizards treated with an adrenal stressor reduced their melanophores reflectance (San Jose et al., 2013). This is also consistent with simulations performed in the ornament system of poikilotherms that accounted for spectral properties of melanin (Grether et al., 2004). However, if melanin alone was responsible for the change in the throat blue colouration, we had expected also a variation in brightness (i.e. Cox et al., 2008). Our experiment of carotenoid extraction from the biopsied skin with acetone demonstrated that a reduction in carotenoid content from the throat blue patch tended to increase the brightness in this patch, whereas the oxidization of the melanin present in the blue patch increased the brightness and decreased the UV-blue chroma here. Therefore, a combined effect of carotenoid allocation and stress-induced melanin deposition may explain the differences in spectral properties in the throat blue-patch between infected and non-infected lizards resulting in a non-significant difference in brightness but a significant increase in the UV-blue chroma from the throat blue patch. Since the colouration in reptiles is the result of the interaction of the light waves reflected/absorbed from the different layers of pigments and structures that compose the dermis of the reptiles (Grether et al., 2004; Kuriyama et al., 2006; Olsson et al., 2013; Soeken et al., 2013), the presence of pigments in the upper layers above the reflective structures of guanine might distort the light reflected from underneath. In addition, those individuals with larger reservoirs of carotenoids might invest these carotenoids to down-regulating the eumelanogenesis in melanophores from the throat blue patch signalizing their ability to cope with oxidative stress (Schantz et al., 1999; Galván and Alonso-Álvarez, 2008; Galván and Solano, 2008, 2009). Indeed, male dominance was related to throat UV-blue chroma in *L. schreiberi* (Martín and López, 2009). However, the individuals with more UV-blue conspicuity in the throat may pay the cost of a higher oxidative stress induced by the synergic effect of testosterone (Alonso-Álvarez et al., 2007) and chronic infections (Atamna and Ginsburg, 1997; Sepp et al., 2012), but also a higher number of agonistic or sexual encounters that might increase the transmission of parasites by contact.

Since the prevalence of intestinal nematodes in this sample was not negligible, nematode presence may represent an important parasite in this population. However, we failed to find a relation between the presence of these parasites and the studied patches here. A plausible explanation to this fact may be that we failed detecting all the nematode-positive individuals due to our diagnosis method that only accounted for the eggs expelled with the feces ignoring the possibility that adult stages inside the lizards passed unnoticed. Alternatively, the parasitosis caused by the nematodes might affect other ornaments in the body of the lizards apart from those measured here.

In conclusion, the balance between melanin and carotenoids allocation in the throat patches of *L. schreiberi* may result in a multicomponent visual signal conveying both aggressiveness and antioxidant competence (Martín and López, 2009). Our hypothesis may explain why brightness has been positively correlated several times with variables of quality and fitness in multiple lacertid species (Martín et al., 2008; Martín and López, 2009, Bajer et al., 2010, 2011; Olsson et al., 2011; Molnár et al., 2012, 2013; Pérez i de Lanuza et al., 2014). However, further studies on pigment composition, and hormonal and oxidative balance in lacertids are desirable to understand how the skin coloration can reflect the trade-offs imposed by the environment.

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A MELANIN-BASED ORNAMENT CORRELATES POSITIVELY WITH PARASITEMIA AND BODY CONDITION IN THE INSULAR SPECIES *GALLOTIA GALLOTI* (SQUAMATA: LACERTIDAE)

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Abstract

Pigment-based traits may act as intraspecific signals used by conspecifics to select mates for good quality genes or to avoid conflicts. The presence or the abundance of parasites in the individuals influences the conspicuousness of these traits. Thus, infections may bias the sexual eligibility or the hierarchic status of the signal bearer towards those individuals that convey competence against parasitic diseases. Herein, we investigated the relation between the parasitemia and a melanin-based ornament present in the cheek (CH) of the dichromatic Canarian lacertid *Gallotia galloti* in a population with high incidence of blood parasites in La Palma. Melanin-based traits were related with the individual quality in some vertebrate groups. In *Gallotia* lizards, UV-blue melanin based ornament has been previously described as a trait involved in sexual selection and hierarchic signalization. Using spectrophotometric techniques, we found that males with more UV-blue chroma in the cheek had better body condition and higher parasite load. Whereas the females that showed more UV-blue chroma had worse body condition than females with more whitish cheeks. In opposition to males, no apparent relation with blood parasite load was found in females. Given the high intracellular oxidative conditions needed for eumelanogenesis, males showing good body condition and strong melanin-based ornaments may signal their ability to cope with oxidant conditions induced by either testosterone or parasitemia. We conclude that this coloured trait was a good predictor of body condition in either sex and a good predictor of blood parasite load in males, indicating that the cheek conspicuousness might be a signal of quality in *G. galloti palmae*.

Keywords: colour, *Gallotia*, Hamilton & Zuk, island, parasite, sexual selection, Zahavi

Introduction

Hamilton and Zuk (1982) proposed a co-evolutionary scenario where individual sexual traits in a population under selective pressure driven by parasitic diseases convey information on the health status of the bearer. This hypothesis assumes the honesty of the secondary sexual traits of the eligible sex (Zahavi, 1975; Grafen, 1990), where the individuals that signal their ability to face parasitic infections may rapidly increase their fitness by means of sexual eligibility by the opposite sex. The Hamilton and Zuk's hypothesis could work if the system of study comply with several requisites: 1) the conspicuousness of an ornament may be sexually dimorphic in the studied species, 2) the sexual trait has to be an honest signal (higher expression in the best individuals and costly to produce and/or maintain), 3) this signal may be perceived by conspecific receptors, and 4) it should occur under a high incidence of chronic parasitic diseases which challenges the production and/or maintenance of the honest signal in the eligible sex.

Most studies concerning the effect of chronic parasite infection on colourful traits were carried out in bird species (reviewed in MØller et al., 1999) where most of the colour characteristics remained in persistent structures (i.e. feathers) (but see Shawkey et al., 2007). In opposition, visual ornaments in reptiles are based on skin structure and pigments directly located in the integument (Grether et al., 2004; Kuriyama et al., 2006; Steffen and McGraw, 2009) which may change in response to rapid physiological reactions (Langkilde and Boronow, 2012) acting as indicators of the individual's current physiological condition (Stapley and Whiting, 2006; Whiting et al., 2006; Font et al., 2009; Bajer et al., 2010, 2011; Molnár et al., 2012; Pérez i de Lanuza et al., 2014). Thus, reptiles may represent good models to test the actual effect of endoparasite infection on the expression of visual ornaments (Ressell and Schall, 1989). Interestingly, few studies found significant relationships between colour showiness on lizard species and their blood parasites (i.e. Ressell and Schall, 1989; Martín et al., 2008; Molnár et al., 2013). In these cases the relationship between the hematic parasites and the colour conspicuousness were in agreement with the predictions of the Hamilton and Zuk's hypothesis (1982) since the brightest individuals showed fewer or no parasitemia. However, Molnár et al. (2013) found that males of *Lacerta viridis* Laurenti 1768 (Squamata: Lacertidae) with more parasitemia were larger and with better condition. The same relation was found in two species of *Podarcis* lizards (Squamata: Lacertidae) (Maia et al., 2014). Because larger lizards in good condition may stand parasitic diseases, these results are in line with the Zahavi's handicap principle where lizards signal their individual quality to stand a handicap (Zahavi, 1975).

Ultraviolet-blue signals in lacertids result from the light reflected by iridophores and melanophores in the dermal layers of reptiles (Grether et al., 2004; Kuriyama et al., 2006; Pérez i de Lanuza and Font, 2010). When the deposition of melanin in the melanophores of the blue patches increases, the relative chroma increases in this area of the spectrum (Grether et al., 2004;

Cox et al., 2008). However, the melanin absorption properties (Ortonne, 2002; Grether et al., 2004) may produce a reduction in the brightness of the coloured patch when its density increases in the melanophores (Ressell and Schall, 1989; Quinn and Hews, 2003). In addition, the structures in the skin of vertebrates, which result in UV-biased ornaments, were proven to be costly to produce and to maintain (Doucet and Montgomerie, 2003; Prum, 2006; Bajer et al., 2011). In fishes, for example, UV reflectance was related with an increase of detection by predators (Modarressie et al., 2013). In birds, the structural plumage colouration was related with blood parasite loads, being the brighter individuals those less parasitized (Doucet and Montgomerie, 2003). Furthermore, UV ornaments in lizards may serve as signals of sexual receptivity and sexual recognition (Le Bas and Marshall, 2000). Indeed, the specific or hierarchic recognition of individuals in *G. gallotia* may drive the gene flow among populations of this lizard species (Thorpe and Richard, 2001). Therefore, UV-biased visual traits may play a role expressing the bearer's genetic quality (Pérez i de Lanuza et al., 2014), and its ability to stand hierarchic, parasitic and predatory pressure.

Lizards of the genus *Gallotia* (Lacertidae: Gallotinae) are well known to bear UV-biased secondary sexual traits (Thorpe and Richard, 2001; Font and Molina-Borja, 2004; Molina-Borja et al., 2006). Particularly, *G. galloti* Oudart 1839 presents UV reflection restricted to the blue patches of the skin while the entire dorsal and lateral background surface of the body exhibits black colouration (Molina-Borja et al., 2004). Therefore, this lizard species is a good model to test the Hamilton and Zuk's hypothesis in relation with UV-blue visual signals since: 1) the species of this Canarian-endemic genus are known to present high prevalence of parasitic infections (e. g. Astasio-Arbiza et al., 1989; Oppliger et al., 1999; Martínez-Silvestre et al., 2001; García-Ramírez et al., 2005; Foronda et al., 2007; and Megía-Palma unpublished data), 2) the colourful patches differ in UV-spectrum reflectance between sexes and among individuals of the same population (Font and Molina Borja, 2004; Molina-Borja et al., 2006; Bohórquez-Alonso and Molina-Borja, 2014), and 3) the male ornamentation is related *in G. galloti* with the reproductive and hierarchical status of the individuals (Thorpe and Brown, 1989; Huyghe et al., 2005; Molina-Borja, 2002; Molina-Borja et al., 2006). In the present study, we tested the relation between the blood parasite load and the conspicuousness of the cheek melanin-based ornament in the individuals from one population of *G. galloti* in La Palma. Based on previous studies, we may expect results either in line with Hamilton and Zuk's hypothesis (1982) or closer to the Zahavi's principle (1975).

Material and Methods

Sampling and collection site

The *tizón* lizard, *Gallotia galloti* (Lacertidae: Gallotinae) is a midsize lizard (in La Palma: male SVL average=107.8; range= 82.7-114 mm; female SVL average= 88.6; range= 74.6-102 mm, after Bischoff, 1982) endemic to La Palma and Tenerife islands in the Canary Archipelago. This is a species of lacertid lizard where the adult males present, to the human eye, cheeks with bright blue colouration and a row of blue eyespots in the lateral and the ventrolateral areas of their body (Font and Molina-Borja, 2004). Although with some seasonal variations (Bohórquez-Alonso and Molina-Borja, 2014), this human-perceived colouration can be observed all year round (R. M.–P. personal observation) probably due to its role in the maintenance of territories to the next breeding season similarly to close related lacertid species (Salvador el al., 1997). *Gallotia* lizards show sexual differences in the UV spectrum of these coloured areas, and males are the showiest ones in this part of the spectrum (Molina-Borja et al., 2006). The adult lizards of this species are mainly herbivorous consuming native and cultivated plant and fruits in the island (see Salvador, 2009). Although the distribution of this species is restricted to two of the seven main islands of the Canarian Archipelago, their populations are not threatened. In fact, in some areas it is considered a plague (Salvador, 1974 and Tello Marquina, 1975 in Salvador, 2009) since these lizards reach very high population densities (Salvador, 2009) and consume the tomatoes, bananas and avocados that are cultivated in the islands.

On March 2014, before the mating season, we sampled lizards of the species *G. galloti palmae* in a single area in La Palma (28.6203,-17.9067), Canary Islands. To capture the lizards, we used a group of eight pitfall traps baited with fruit and tomato (Oppliger et al., 1999). These traps were located in an area of 200 square meters among banana crops. Traps were hidden in the bushes or placed on the ground next to the walls of the contiguous plantations, where lizards use to bask, and always out of the direct sunlight to avoid suffocation of the lizards. The traps were surveyed every 15 minutes and the lizards inside were collected and transported in individual cotton bags which allowed good aeration. We collected 40 adult lizards, 17 males and 23 females. Collecting adults may be important since only adult individuals in this species may display full colour signals (Thorpe and Richards, 2001). All the lizards were measured and sampled in a darkened room as indicated below and they were successfully released at the same spot where they had been captured within the next 24 hours of collection. Each lizard was measured to the nearest millimetre with a ruler. The mean snout to vent length (SVL) ± standard error of these lizards was 111.4 ± 1.5 mm for males, and 96.9 ± 1.5 mm for females. Also, we weighted the individuals to the nearest gram with a digital balance. The mean weight ± standard error of these lizards was 48.7 ± 2.6 g for males and 29.8 ± 1.8 g for females. The body condition index (BCI) was later calculated using the residuals of the correlation between SVL and weight (see Schall and Pearson,

2000 but also Green, 2001). Because 17 of the lizards showed their tail regenerated and not all individuals regenerate it to its original size in presence of parasites (Oppliger and Clobert, 1997), we included the total length of each lizard as a controlling variable in the calculation of the individual BCI.

Measurement of the cheek (CH) reflection

We measured the cheek colourful patch (Figure 1a) of each of the 40 lizards three consecutive times. We selected the colourful patch in the cheek (CH) because this trait is related with the quality of the individual (Huyghe et al., 2005) and is involved in the sequential assessment game during male-male interactions in *Gallotia galloti* (Molina-Borja et al., 1998, 2002). Since this lizard species is sexually dimorphic for this trait we measured the same area in both sexes (Figure 1). We measured the reflectance spectra of this blue ornament from 300 to 700 nm with a spectrophotometer (Jaz DPU® Module) with a Pulsed Xenon Light Source (Jaz-PX) connected to an optical fiber. The probe was mounted within a holder that ensured readings were taken from areas 1 mm in diameter at a constant distance of 3 mm from the skin surface with a 90º angle (Endler, 1990; Martín and López, 2009; Bajer et al., 2010; Pérez i de Lanuza and Font, 2010). The measured spectrum covered the broadest range of wavelengths known to be visible to lizards (Elligson et al., 1995; Fleishman et al., 1993, 1997; Loew et al., 2002; Bowmaker et al., 2005; Macedonia et al., 2009; Pérez i de Lanuza and Font, 2010). All the measurements were relative to a 99% WS-1 white reflectance standard (all the components from Ocean Optics Inc., Dunedin, FL, USA). The spectral records were made in a darkened room to avoid that environmental light could affect the data.

Blood parasites

We bled each lizard at the base of the tail with a sterilized needle (Megía-Palma et al., 2013 and 2014). In the case of males, we carefully avoided the area of the hemipenes by bleeding the tail at least 4 cm from the cloaca and always in a narrower area than that where the hemipenes were located. The blood drop obtained by this method was collected with a heparinized capillary (BRAND, micro-haematocrit tubes, 75 x 1.1 mm, Na-heparinized). With this blood sample we made a thin layer blood smear to count the number of blood parasites per 5000 red blood cells. With this purpose we fixed the dried blood smears with methanol (Rogier and Landau, 1975) and we stained them during 40 minutes with Giemsa diluted 1:10 in buffer, pH 7.2 (Schall, 1986). Then, we counted 5000 erythrocytes for each smear in search of blood parasites at 1000 magnification in an area with homogenous distribution of red blood cells (Merino and Potti, 1995) with a microscope BX41TF (Olympus, Tokyo, Japan).

Statistical analyses

We analysed the spectral data obtained from the right cheek of the lizard by the segment classification analysis (Endler, 1990). This method divides the spectrum in ranges of 75 nanometers which approximately correspond with the colours blue –UV-blue–, green, yellow and red. In an exploratory analysis of the spectral data we found a high correlation ($r^2 \sim 0.90$; $p < 0.0001$ for either sex) between the relative chroma from 300 to 475 nm calculated as $R_{300-475}/R_{300-700}$ and the relative chroma from 625 to 700 nm calculated as $R_{625-700}/R_{300-700}$ (Endler, 1990; Grill and Rush, 2000; Pérez i de Lanuza and Font, 2010; Deitloff et al., 2013; Pérez i de Lanuza et al., 2014). Therefore, we calculated and index of "blueness" dividing the relative UV-blue chroma by the relative chroma between 625 and 700 nm. In this way we calculated the proportion of UV-blue light that accounted for the total spectrum in each lizard (hereinafter referred as chroma for simplicity). Furthermore, we calculated the brightness for the cheek spectra as $\Sigma Q_{300-700}$ (Montgomery, 2006; Pérez i de Lanuza et al., 2014). In addition, we calculated the hue of the spectrum as the value at λ of Q_{max} (Montgomery, 2006). The distribution of the residuals of the models for chroma and brightness were visually explored for normality and homocedasticity. However, even after log-transformation of the hue neither the variable, nor the residuals of the model were normal. Thus, we performed non parametric Spearman's partial correlations with this variable.

To study the relation between cheek brightness and chroma with the BCI, and the parasite load, we performed a set of ANCOVAs in Statistica 10.0 (Statsoft, Inc.). In each ANCOVA the variables of colour: the brightness and the chroma were the dependent variables, whereas the sex of the individuals was set as a factor, and the body condition, and the blood parasite load were set as independent variables in the analyses. Because we were interested in sexual differences in these relations, we included the interaction between the sex and the independent variables. To accomplish normality and homocedasticity of the final model we log-transformed the dependent variables and the variable of parasite load (Molnár et al., 2013).

Results

Parasite infection

We found 39 out of 40 (97%) individuals infected with blood parasites. Although parasite load did not differ significantly between sexes ($F_{1, 38}$ 1.4; $p = 0.2$), the mean ± standard error infection per 5000 red blood was higher in males (98 ± 26.2; range= 1-338) than in females (65.8 ± 23.8; range= 0-556). Only one type of hemoparasite was found infecting lizards. Mature and immature parasite stages were found infecting red blood cells in peripheral blood. The mean size of the parasite hematic stages (N=49) was 9.1 ± 0.15 µm in length (range: 5.9-10.9), and 2.1 ± 0.07 µm in width (range: 1.1-3.5). The overall morphology and the fact that the parasite distorted the

nuclei of the blood cells (Figure 2a) make likely that this parasite belonged to the genus *Karyolysus* sp. (Apicomplexa: Adeleorina) as it has been proposed for hematic parasites described infecting *G. bravoana* in an adjacent island (Martínez-Silvestre et al., 2001). No significant relation was observed between BCI and parasitemia ($F_{1, 38}$ 1.1; p= 0.3).

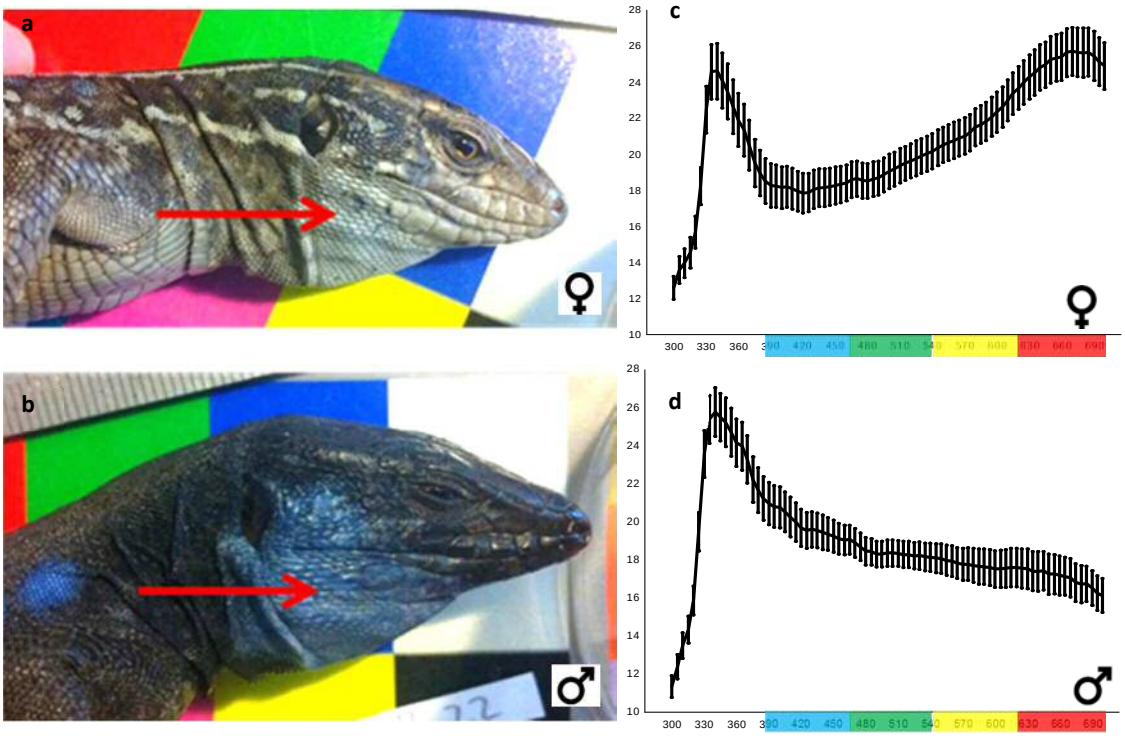

Figure 1. (a) Female (above) and **(b)** male (below) *tizón* lizard showing the cheek ornamentation area measured with spectrophotometry. **(c)** Mean ± SE (showed at 5 nm intervals) reflectance spectra of the right cheek of females and **(d)** males of *Gallotia galloti palmae*.

Cheek reflectance

The chroma calculated as the relation between the relative chroma from 300 to 475 nm and the relative chroma from 625 to 700 nm, was significantly explained by the interaction between the sex of the individuals and the parasite load ($F_{1, 34}$= 5.5; p= 0.02). Particularly, male lizards that showed cheek patches with higher values of chroma had more blood parasites, but the females did not show a significant relation between the cheek chroma and the parasite load (Figure 2b). In addition, the interaction between the sex of the individuals and the BCI was significant ($F_{1, 34}$= 14.3; p< 0.001). Males with better body condition showed more saturated bluish cheeks (Figure 2c), whereas females with better body condition showed cheeks with less proportion of UV-blue light (Figure 2d). No relation was found between the cheek brightness and the BCI ($F_{1, 34}$= 0.06; p= 0.8) or the parasitemia ($F_{1, 34}$= 0.3; p= 0.6). The hue was only correlated with the sex

(Spearman r= 0.55; p< 0.001). Indeed, the females showed whitish to greyish cheek ornaments (Figure 1b) that fairly differed from the blue colouration of male cheeks.

Discussion

In this study we explored the relation between a melanin-based trait, the body condition and the parasite load in a dichromatic lizard species. In compliance with the Zahavi's principle (1975), the conspicuousness of a sexual ornament may mirror the cost of its production or maintenance. In La Palma, the chroma of this trait was significantly related with the body condition in either sex of *G. galloti* suggesting that cheek coloration is a condition-dependent trait in this lizard species. In addition, the males from La Palma that showed cheeks with higher UV-blue chroma and better body condition had also higher blood parasite load. Thus, the interaction between parasites and UV-blue ornaments in lacertids may not be obvious. The main type of melanin in the melanophores of reptiles is the eumelanin (but see Roulin et al., 2013) and its synthesis is favoured under hormonal induction (Quinn and Hews, 2003; Ludwig et al., 1998), or low bioavailability of reduced glutathione (GSH). The GSH is the primary antioxidant molecule in eukaryotic cells (Meister, 1994) and it is implied in practically all major biological processes such as signal transduction, gene expression or apoptosis (see Sies, 1999). It is well known that low levels of GSH favours high oxidant conditions (Galván and Solano, 2009, 2015). Therefore the reduction in glutathione availability, needed for eumelanogenesis, may handicap the individuals bearing eumelanin-based ornaments (Galván and Alonso-Álvarez, 2008). Paradoxically, the males of *G. galloti* from La Palma showed better body condition when the melanin-based ornament of their cheek was more conspicuous. Previous evidences suggest that melanin-based traits may signal male individual quality in lizards (Bajer et al., 2010, 2011; Vroonen et al., 2013; Pérez i de Lanuza et al., 2014). In addition, it can also signal the bearer's capability to mobilise other antioxidants as has been shown in other vertebrates (Galván and Alonso-Álvarez, 2008). In this sense, chicks of *Parus major* Linnaeus 1758 treated with DL-buthionine-S, R-sulfoximine, an inhibitor of GSH production, increased the area of a melanin-based trait and compensated the decrease in GSH levels increasing the levels of circulating carotenoids (Galván and Alonso-Álvarez, 2008).

Parasites may act as primary triggers or enhancers of oxidative stress (Mougeot et al., 2009) depleting the GSH availability in their hosts (Atamna and Ginsburg, 1997; López-Arrabé et al., 2015). In this sense, adeleorine parasites may increase both the blood cell regeneration rate (Martínez-Silvestre et al., 2011 in Martínez-Silvestre and Arribas, 2014) and the basal metabolism in lizards (Schall, 1986). An increase in cell metabolism may lead to the increase of pro-oxidant reactive species (Finkel and Holbrook, 2000). Hence, this fact might explain the positive correlation between parasite load and the melanin-based colouration in the cheek of male lizards in this population. In addition to the low levels of GSH needed for eumelanogenesis, the

testosterone may also play a role in the deposition of eumelanin in the skin of lizards (Quinn and Hews, 2003; Cox et al., 2005, 2008). This steroid hormone is known to drive processes of oxidative stress and immune constraints (Folstad and Karter, 1992; Marler et al., 1995; Alonso-Álvarez et al., 2007). Therefore, a combined effect of testosterone levels and parasite load may induce the synthesis and deposition of melanin in the dermal melanophores of lizards. Thus, male *tizón* lizards with better body condition may signal through the cheek ornaments the bearer's capability to cope with oxidative stress.

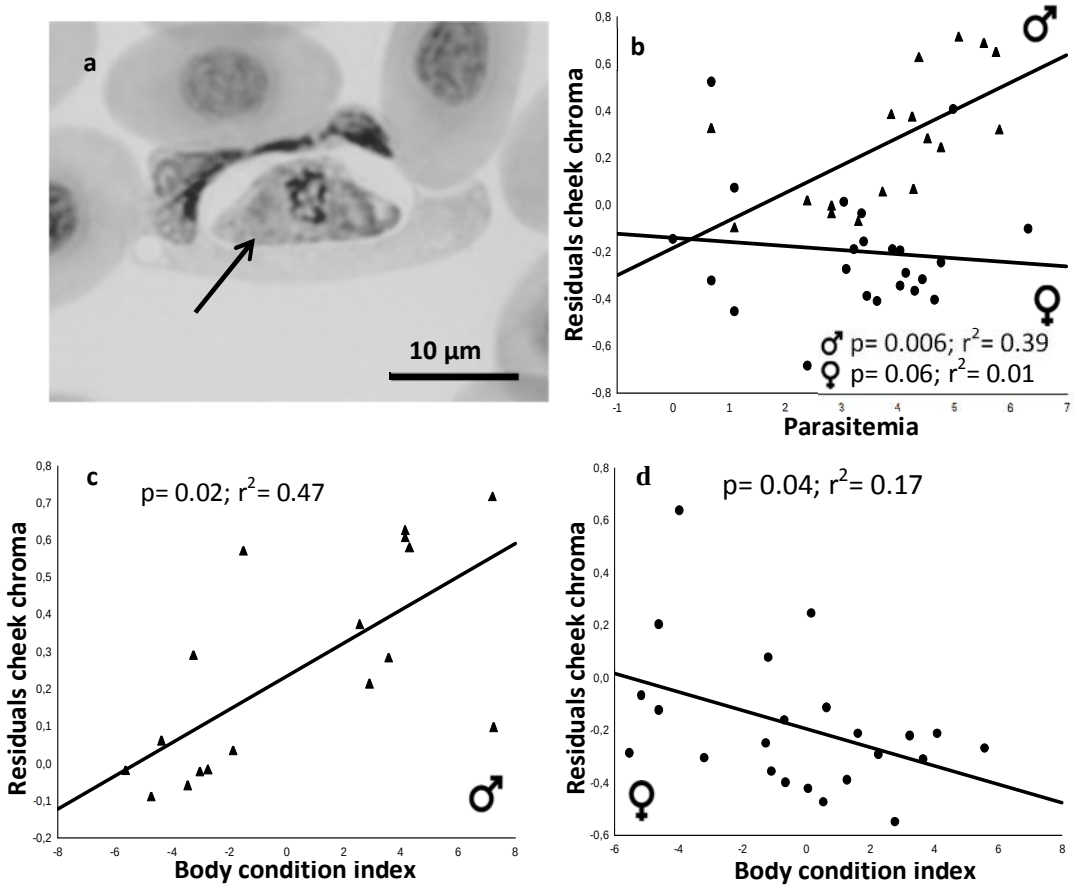

Figure 2 (a). An enlarged *G. galloti* red blood cell with distorted nucleus parasitized by a gamont of *Karyolysus* sp. (black arrow). (b). Relationship between the parasitemia and the residuals of cheek chroma controlled by the body condition in males (triangules) and females (dots). Relationship between the body condition and the residuals of cheek chroma controlled by parasitemia in males (c) and females (d)

In the present study, the results achieved from the males of *G. galloti palmae* are compatible with previous studies using similar methods in close related host-parasite models. For example, Molnár et al. (2013) quantified the nuptial UV-blue throat colour of male *L. viridis* (Squamata: Lacertidae) by spectrophotometric techniques and made similar calculations of the spectral properties than we use in the present study. They found a positive relation between the Adeleorine

blood parasite load and the body condition of the male lizards, and a negative relation between the parasite load and the brightness of the nuptial UV-blue throat of the male lizards (Molnár et al., 2013). Nevertheless, these results are compatible with the stimulation of eumelanogenesis in the nuptial melanin-based ornaments of lacertids favoured by oxidative stress since the increase in melanin density may reduce the brightness, or increase either the chroma or the hue in melanin-based traits of lizards (Cox et al., 2008). In other study, bluer males of the Aruban whiptail lizard, *Cnemidophorus arubensis* Lidth de Jeude 1887, were more likely to have blood cells infected by gametocytes of an Adeleorine parasite than dull blue or brown males (Schall, 1986). However, in that study an observer scored the dorsal patterns of the male individuals.

On the other hand, although the cheek coloration of females did not show a significant relation with the parasite load, there is a significant relation between values of chroma and body condition in females indicating that those with higher values of chroma in cheeks showed worst body condition. These results suggest that cheek ornaments are a condition-dependent trait in the females of *G. galloti palmae* and might serve also as a good predictor of female individual quality. The reflectance in long wavelengths (above 575 nm) is associated with the light reflected by the connective tissue underlying the melanophores (Grether et al., 2004; San Jose et al., 2013). Since the deposition of melanin in the melanophores may be driven by androgen control (Cox et al., 2005, 2008) sexual difference in the production of testosterone may induce the sexual differences in melanin allocation and cheek colouration in *G. galloti*. Thus, females with better body condition reflected more from the dermis background. The connective tissue underneath the different layers that produce the colour effect in the skin of lizards is associated with the metabolism of the vitamin A (Grether et al., 2004; San Jose et al., 2013). The role of pro-vitamin A of some carotenoids is well characterized (Goodwin, 1986 in Grether et al., 2004). Thus, the females that show cheeks with higher proportion of longer than shorter wavelengths might be signalling their ability to get these antioxidants from the environment (von Schantz et al., 1999). Ornaments related with the capability to get antioxidants has been previously associated to reproductive investment (Weiss et al., 2011) and offspring survivorship (Weiss et al., 2009) in lizard species where females were ornamented, and hence the eligible sex. Therefore, the correlations between the cheek colour and the body condition in either sex suggest a bidirectional selection in *G. galloti palmae* that may favour dichromatic differences between genders.

In conclusion, the relations found here between the conspicuousness of the cheeks of *Gallotia galloti* from La Palma, their body condition and the parasite load are compatible with the hypothesis of the melanin-based ornaments signalling the individual capability to cope with oxidative stress in line with Zahavi's handicap principle. Although sexual differences in parasitemia were not significant, the higher parasitemia in males hints the testosterone driven dichromatic differences in this species (Folstad and Karter, 1992). In addition, the cheek chroma

can be considered a good predictor of parasite load and body condition in male *tizón* lizards from La Palma. Likely, this trait might be a signal of quality in *G. galloti palmae*.

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INFECTION BY COCCIDIAN PARASITES IS RELATED TO VARIATION IN CHROMATIC DIMORPHISM OF THE COAST RANGE FENCE LIZARD, *SCELOPORUS OCCIDENTALIS BOCOURTII* (SQUAMATA: PHRYNOSOMATIDAE)

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Abstract

Chromatic dimorphism driven by sexual selection is common in species evolving in a context of high pressure due to parasitism. Showy color patches of phrynosomatid lizards can be condition-dependent but they usually convey different information depending on the sex of the bearer. In males, colorful ornaments have been related with the maintenance of a territory, aggressiveness or health status, whereas in females the role of colorful ornaments is related with the reproductive investment of the bearer and sexual receptivity. In a population of the Coast Range fence lizard with high incidence of parasites of the genera *Acroeimeria* and *Schellackia* in California, we quantified spectral data on ventral blue and yellow color patches implied in social interactions. In this population, individuals of either sex were ornamented and the relations found between the conspicuousness of their ventral color patches and the coccidiosis studied here suggest that parasites influence the chromatic dimorphism in this population. Indeed, the hue and the chroma of the ventral blue patch differed between males and females that were not infected by intestinal parasites of the genus *Acroeimeria*. However, the sexual differences were not significant between infected individuals. As opposed to this, the infected males and females differed in the brightness of their condition-dependent yellow patch on the forelimb, but the sexual difference was not significant between uninfected individuals. Additionally, the infection by hemococcidia of the genus *Schellackia* affected significantly more males than females. Those males infected by *Schellackia* parasites showed darker (lower brightness) blue ventral patches and more saturated yellow patches on the forelimbs. The results suggest a differential role for parasites in the color ornaments of male and female Coast Range fence lizards with hypothetical implications on sexual and territorial signaling.

Keywords: Hamilton and Zuk, ornaments, parasites, reptile, visual signals, western fence lizard, Zahavi.

Introduction

The handicap principle (Zahavi, 1975) proposed an explanation for the maintenance of exaggerated traits in ornamented species. The genes implied in the expression of these traits may be transmitted to the offspring by a mechanism of sexual selection because the individuals expressing these characters transmit their genetic quality to produce or maintain the ornament (e.g. Sinervo and Lively, 1996). Hamilton and Zuk (1982) proposed that parasites influence the conspicuousness of the sexual traits of their hosts. Thus, these traits can be used as honest signals by conspecifics to make decisions during rival (Stapley and Whiting, 2006) or mate assessment (Baird, 2004) biasing the selection towards individuals with better resistance to diseases. Commonly, in nature, exaggerated or conspicuous traits are found in only one sex. However, species where both sexes are similarly ornamented offer a good opportunity to study the role of parasitic diseases on the conspicuousness of ornamental traits in both sexes.

The Coast Range fence lizard (*Sceloporus occidentalis bocourtii*) (Squamata: Phrynosomatidae) is a subspecies of the Western fence lizard (*S. occidentalis*). This is a polygynous territorial phrynosomatid lizard that hosts multiple endoparasites (Bonorris and Ball, 1955; Bovee and Telford, 1965; Dunlap and Schall, 1995). The home ranges of both female and male overlap (Sheldahl and Martins, 2000) which motivates social interactions. During social interactions the behavioral display of the individuals (Cooper and Burns, 1987; Sheldahl and Martins, 2000; Stebbins and McGinnis, 2012) enhances the visibility of their ventral colorful patches conveying different information depending on the sex of the bearer. Specifically, the colorful patches in male phrynosomatids were good predictors of infection by parasites (Ressel and Schall, 1989), dominancy status (Meyers et al., 2006; Langkilde and Boronow, 2012), and territoriality (see Moore and Marler, 1987; Rand, 1992; Smith and John-Alder, 1999).

The role of ornaments in females is still under debate (Amundsen et al., 1997; Amundsen, 2000; Ord and Stuart-Fox, 2006) and whether these ornaments are expressed in females due to genetic correlation with males (e.g. Lande, 1980) or due to direct selection on females (e.g. Chan et al., 2009) may depend on the biological system studied. However, there is quite a bit of evidence for female color functionality in lizards (Watkins, 1997; LeBas and Marshall, 2000; Ord and Stuart-Fox, 2006; Olsson et al., 2013). In female phrynosomatids, colorful ornaments were correlated with body condition (Weiss, 2006), parasite load (Weiss, 2006; Calisi et al., 2008), reproductive investment (Sinervo, 2001; Weiss et al., 2009; Weiss et al., 2011), sexual recognition or sexual receptivity (Cooper, 1984; Cooper and Burns, 1987; Cooper and Crews, 1987; Cooper, 1988; Calisi and Hews, 2007). Indeed, when female fence lizards reject a candidate male they laterally flatten and display push-ups, similarly to the territorial behavior of males, enhancing the visibility of their patches (Figure 1; Cooper and Burns, 1987). This rejecting behavior of the females may

reduce the number of male mating attempts in fence lizards (Cooper and Burns, 1987) reducing the energetic costs linked to reproduction for females (Cooper and Crews, 1987; Olsson, 1995; Ruiz et al., 2011). Likely, expression of ornaments in females depends on condition or stress and might evolve through male mate selection as long as this selection conferred an advantage to the offspring (Weiss, 2006; Chan et al., 2009; Weiss et al., 2009 and 2011).

Figure 1. Sceloporine lizards during social displays stand up on two or four of their limbs and flatten their bodies making visible their ventral color patches. Photo by Maggie Smith taken from www.flickr.com /photos/ slomaggie/ 6948732194

The aim of this investigation is to study the conspicuousness of the ventral patches of *Sceloporus occidentalis bocourtii* Boulenger, 1885 in relation with the incidence of infections by two different coccidian parasites. Because the colorful patches in *Sceloporus* may convey different information depending on the sex of the bearer (Cooper and Burns, 1987), we also expected a different phenotypical response to infections depending on the gender of the host.

Material and Methods

Sampling and collection site

In May of 2014 we collected 68 individuals of *S. occidentalis bocourtii* using a slip noose attached to the end of a fishing pole (e.g. Schall and Marghoob, 1995) in a linear transect of 400 meters (from 36.985270,-122.061440 to 36.985287,-122.056934) in the campus of the University of California in Santa Cruz (UCSC). The lizards were transported in a cooler to the lab in the UCSC facilities to perform all the color measurement under standardize conditions of light (see below). The snout-to-vent length (SVL) for each lizard was measured to the nearest millimeter with a ruler. Also, we weighted the individuals to the nearest centigram with a digital balance. The body condition index (BCI) was later calculated using the residuals of the regression of log weight on log SVL (Dunlap and Mathies, 1993; Schall and Pearson, 2000 but see Green, 2001). The sex of the individuals was determined by the presence of enlarged post-anal scales (Parker,

1994; Cox et al., 2005; Langkilde and Boronow, 2012). No lizard suffered damage during the manipulations in the lab and they were released to the same spot where they had been caught.

Survey of blood smears

We bled the base of the tale of each lizard with sterilized needles (Megía-Palma et al., 2013 and 2014). In the case of male lizards, we carefully avoided the area of the hemipenes by bleeding the tale at least 2 cm from the cloaca and always in a narrower area than that where the hemipenes are. The drop of blood obtained by this method was collected with a heparinized microcapillary (BRAND, micro-haematocrit tubes, 75 x 1.1 mm, Na-heparinized). With this blood sample we made a thin layer blood smear, then we fixed the dried blood smears with methanol and we stained them for 40 minutes with Giemsa 1:10 at pH 7.2 (Svahn, 1975). Following the methods described in Merino and Potti (1995), we screened 15.000 red blood cells of each individual lizard at 1000X magnification (Megía-Palma et al., 2014) diagnosing the presence of hemoparasites in the blood for each infected lizard.

Survey of fecal samples

Fecal samples were collected directly into 1.5mL micro centrifuge tubes by massaging briefly the belly of the lizards. These fecal samples were stored in 1 mL of potassium dichromate (Duszynski and Wilber, 1997). For the microscopy screening of the fecal samples the standard protocol of concentration of parasites by means of Sheather's sugar flotation technique (Levine, 1973) was followed which allowed us to recover coccidian oocysts (Duszynski and Wilber, 1997). Each sample was screened at 600X magnification lens with the same optic microscope that was used for screening the blood smears. Our aim was to evaluate the presence of intestinal coccidia which have been previously described to infect this lizard species (Bovee and Telford, 1965; Clark, 1970). Using the above mentioned technique, the presence of intestinal coccidia for each individual lizard was diagnosed.

Measurement of the color patches reflection

We measured the reflectance from the blue patch on the right side of the belly; and the yellow patch located on the anterior part of the right forelimb (Figure 2a). We selected these patches because they are known to convey information to intra- and intersexual receivers in several species of the genus *Sceloporus* (Cooper and Burns, 1987; Weiss, 2006; Weiss et al., 2009; Stebbins and McGinnis, 2012; Bastiaans et al., 2013). All the spectral measurements of the colorful patches were obtained by spectrophotometry from 400 to 700 nm in order to find perceivable patterns in the human range of vision (Endler, 1990). Lizards can perceive spectral light in the ultraviolet range (Fleishman et al., 1993). However, the role for UV in the colorful

patches in lizards of the genus *Sceloporus* remains to be clarified, since UV reflectance within species of the genus *Sceloporus* may be weak (Stoehr and McGraw, 2001; Langkilde and Boronow, 2012). The spectrophotometer, an USB2000 Ocean Optics, was connected to a fiber-optic probe (Ocean Optics Inc., Dunedin, FL, USA). The light source used was a deuterium-tungsten light (MINI DT1000A-112) (Analytical Instruments System, Inc., Ringoes, NJ, USA). In a darkened room, we measured the reflectance from the colorful patches with a probe at 45° of inclination and a constant distance of 3 mm from the skin surface repeating the readings three consecutive times. All the measurements were relative to a 99% WS-1 white reflectance standard.

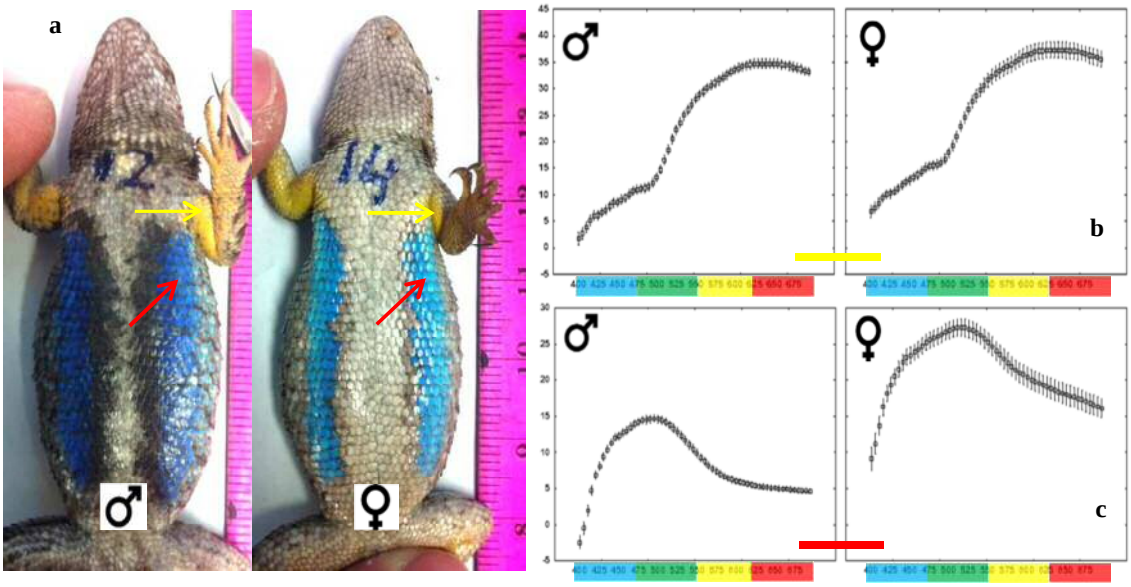

Figure 2. (a) Male and female *Sceloporus occidentalis bocourtii* showing their ventral ornamentation. (b) Spectra from the forelimb yellow patch (yellow arrow in (a)). (c) Spectra from the blue ventral patch (red arrow in (a)).

Statistical analyses

We analyzed the spectral data from the blue and the yellow patches of the lizards by the segment classification method, which assign to each color (blue, green, yellow and red) ranges of 75 nanometers for the human visual spectrum (Endler, 1990; Grill and Rush, 2000). Thus, the total brightness for each spectrum was calculated as ΣQ_T with Q being the percentage of reflectance for a given wavelength (λ), whereas the hue was calculated as the value of λ for the Q_{max} (i.e. λ_{max}; Montgomery, 2005). Whereas we calculated the relative chroma for the specific segment defined above as $\Sigma Q_{segment}/\Sigma Q_T$.

In a preliminary analysis, we found a strong negative relation between the relative chroma in the blue range (from 400 to 475 nm) and the relative chroma in yellow range (from 550 to 625 nm) in the blue patch of both males and females ($p < 0.00001$). Thus, for the blue patch we used the

relation between the relative chroma in short wavelengths divided by the relative chroma in mid-to-long wavelengths as a value of chroma.

We tested for sexual differences in the color of the two patches considering the presence and absence of intestinal coccidiosis (i.e. *Acroeimeria*) by running ANCOVAs in Statistica 10.0 (Statsoft Inc.). The factors of the analyses were the sex of the individual, and the presence of intestinal coccidia. The interactions sex*BCI, and sex*presence of intestinal coccidia were included in the analyses. Furthermore, we included the presence of hemococcidia as a cofactor in the analyses. The dependent variables were transformed by the Johnson's transformation when their distribution differed from normality. The variable BCI of the individuals was included as co-variable in all these analyses. Then, we ran Fisher's least significance difference test (LSD) post-hoc analyses to reveal intra- and intersexual differences in coloration between infected and non-infected lizards (Dunlap and Schall, 1995).

As we found parasites in the blood of only two females we tested for the effects of the infection by hemococcidia considering only males in the analyses. We compared infected and non-infected males for each colored patch. We included in all the analyses the presence/absence of intestinal coccidia as a cofactor and the BCI of the individuals as co-variable. The variable of brightness from the blue ventral patch and the hue of the yellow patch had non normal distribution even after the transformation so differences in these variables between infected and non-infected lizards were analyzed using non-parametric Mann-Whitney *U*-test.

Results

Morphology and parasitic infections in the lizards

The mean snout to vent length (SVL) ± standard error of the lizards in the sample was 59.4 ± 0.95 mm for males, range= 41.0 to 69.0 (N=45), and 56.4 ± 1.09 mm for females, range= 48.0 to 68.0 (N=23).The mean weight ± standard error of these lizards was 8.05 ± 0.32 g for males, range= 3.03 to 12.46, and 7.4 ± 0.50 g for females, range= 3.5 to 12.77.

In relation with intestinal coccidia (Figure 3a), we did not find sexual differences in infection between the 23 Coast Range fence lizards infected (33.8%): 14 out of 45 (31%) males and 9 out of 23 (39%) females were infected ($\chi^2_{1,\ 69}$= 0.3; p= 0.6). We compared the morphology of the parasite found in the fecal samples of the lizards from this population with the previously described species of coccidia in this host species (Megía-Palma et al., 2015) and accordingly it was identified as *Acroeimeria sceloporis* Bovee and Telford, 1965 (Apicomplexa: Eimeriidae). Additionally, the morphology of the parasites found in erythrocytes in the blood of the individuals of this population of *S. occidentalis* matched with the former description of the hemococcidian

Schellackia occidentalis (Apicomplexa: Schellackiidae) (Bonorris and Ball, 1955; Telford, 2008) in the same host species from California (Figure 3b). Specifically, we found 21 of 68 (30.8%) individuals infected by these hemococcidia. Males were significantly more often infected than females ($\chi^2_{1, 69}$= 7.7; p= 0.005; prevalence of infection in males= 19/45 (42.2%); prevalence of infection in females= 2/23 (8.7%)).

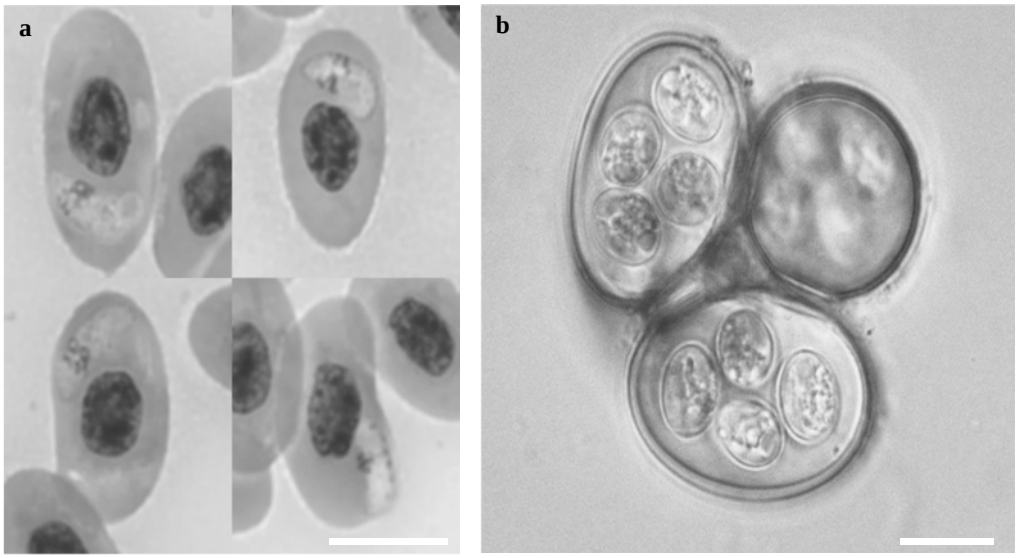

Figure 3. (**a**) Sporozoites of *Schellackia occidentalis* in the blood of *S. occidentalis bocourtii*. (**b**) Oocysts of *Acroeimeria sceloporis* in the fecal samples of these lizards. Scale bars= 10 µm.

Correlations between parasitic prevalence and patch conspicuousness

In relation with the blue ventral patch, the ANCOVA revealed that the interaction between sex and the presence of the intestinal coccidia, *Acroeimeria sceloporis*, explained the intra- and intersexual variation in the chroma ($F_{1, 61}$= 4.9; p= 0.03) of the blue ventral patch. The males not infected by *A. sceloporis* showed blue ventral patches with significantly more chroma than infected males or females infected or not (Figure 4a; Fisher's LSD post-hoc Table 1). In addition, the interaction between sex and the presence of *A. sceloporis* also explained the variation in the hue of the blue ventral patch ($F_{1, 61}$= 4.4; p= 0.04). Hue of uninfected females differed significantly from males infected or not and infected females differed from uninfected males (Figure 4b; Table 1). However, no significant differences were observed between the infected individuals of both sexes (Table 1). On the other hand, males not infected by *Schellackia occidentalis* showed brighter blue ventral patches than infected males (Figure 4c; U= 111.0, Z= 3.11, p= 0.001). Similarly, the correlation between brightness of this patch and BCI was tested and was found to be not significant (Spearman's correlation: p> 0.05).

In relation with the yellow patch in the forelimbs, the males infected by *Schellackia occidentalis* showed significantly higher values of chroma than the uninfected males (Figure 4d; $F_{1, 41}$= 5.7; p= 0.02). Furthermore, we observed a significant negative relation between the brightness of yellow patch and the body condition of the individuals independently of their sex (Figure 4e; $F_{1, 61}$= 4.8; p= 0.03). In addition, the differences that we found in the brightness of yellow patch were also explained by the presence of the intestinal coccidia *A. sceloporis* (Figure 4f). Specifically, the brightness of the yellow patch on the forelimb was explained by the interaction between sex and the presence of *A. sceloporis* ($F_{1, 61}$= 4.4, p= 0.04). The Fisher's LSD posthoc revealed that uninfected males and females did not differ in the brightness of the forelimb. However, infected males and females significantly differed in brightness of the forelimb. Infected females showed brighter forelimbs than males, infected or not, and infected males had darker forelimbs than uninfected females (Table 1). The remaining relations tested in this study were not significant (P> 0.05), hence are not shown.

Discussion

The relations found between the coccidian species studied and the conspicuousness of the ventral color patches of *S. occidentalis bocourtii* suggests a role of parasites in the visual signaling of this polygynous lizard species. However, various spectral properties of the yellow and the blue patches were differently related to the presence of parasites of the genera *Acroeimeria* and *Schellackia*. Similar evidence for the effect of different types of parasites on ornamental patches that are based on different pigments had been demonstrated in birds (McGraw and Hill, 2000; Fitze and Richner, 2002). In this study, the infection by *Acroeimeria* parasites was related to the loss of chromatic dimorphism of the ventral blue patch. The values of hue and chroma of the blue patch from uninfected individuals of either sex indicated chromatic dimorphism in this species. The uninfected males exhibited higher chroma and lower values of hue than the uninfected females. However, the sexual differences in chroma and hue of the blue patch were not significant when individuals passing oocysts of *Acroeimeria* in their feces were compared. These results suggest the loss of chromatic dimorphism in the blue patch associated to the infection by *Acroeimeria* parasites. The sex of *Acroeimeria*-free lizards might be easily recognizable by the opposite sex because uninfected males and females significantly differed in chroma and hue of the blue patch.

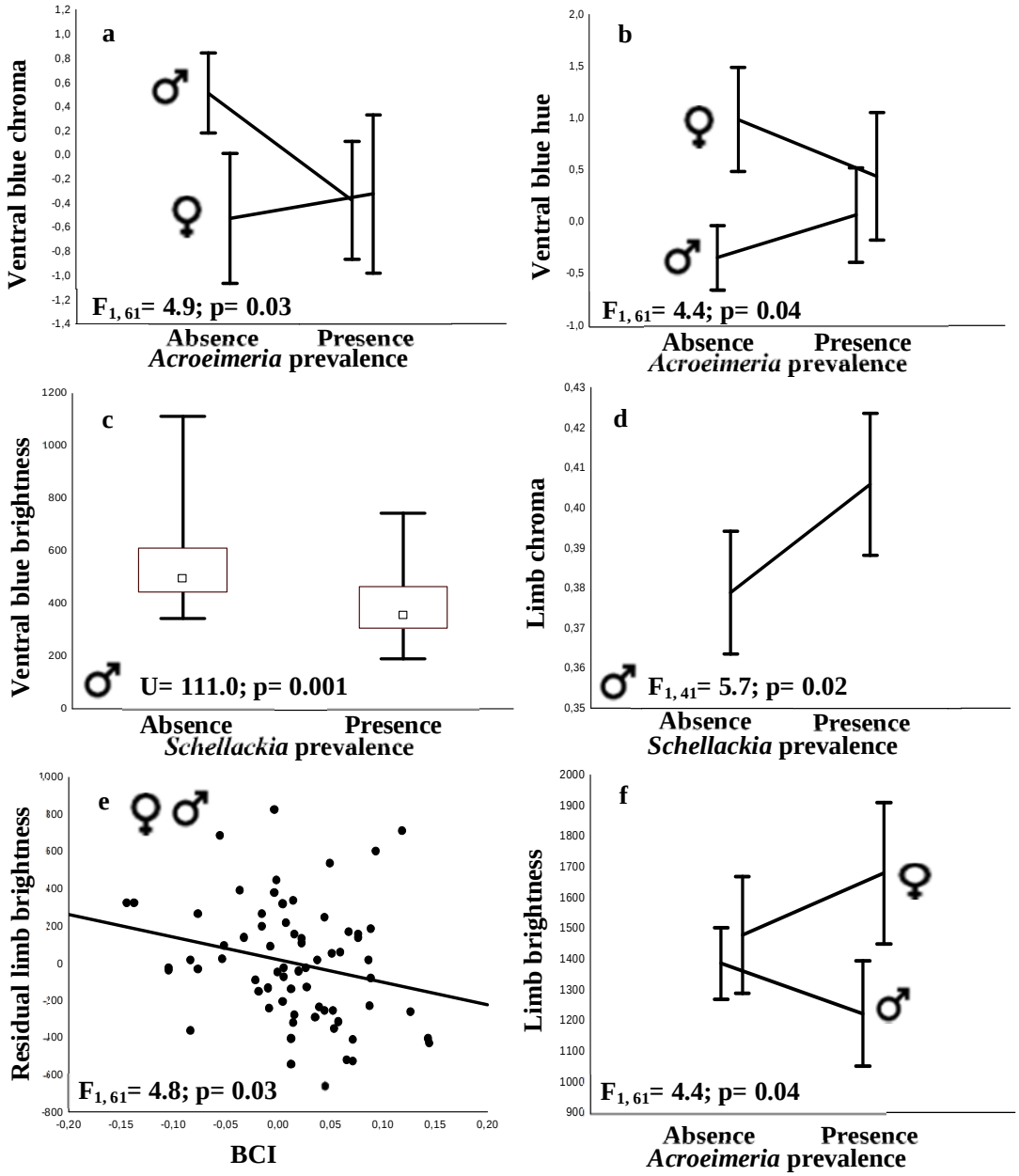

Figure 4. Intra- and intersexual differences (mean ± confidence interval) in chroma (**a**) and hue (**b**) of the ventral blue patch considering the presence of *A.sceloporis*. (**c**) Differences in brightness of the ventral blue patch in males uninfected and infected by *Schellackia occidentalis*. (d) Differences in chroma of the forelimb patch between males uninfected and infected by *S. occidentalis*. (**e**) Relation between the brightness in the yellow patch and the body condition. (**f**) Intra- and intersexual differences (mean ± CI) in the brightness of the forelimb yellow patch considering the infection by *Acroeimeria sceloporis*.

Table 1. Post hoc Fisher's LSD for the ANCOVA of the interaction sex*infection by *Acroeimeria sceloporis* over the different spectral properties of the blue ventral patch (BVP) and the forelimb patch (FL).

		♂ Infected	♀ Uninfected	♀ Infected
BVP_chroma	♂ Uninfected	**0.0035**	**0.0009**	**0.03**
	♂ Infected		0.7	0.8
	♀ Uninfected			0.5
BVP_hue	♂ Uninfected	0.12	**0.000001**	**0.007**
	♂ Infected		**0.002**	0.2
	♀ Uninfected			0.11
FL_brightness	♂ Uninfected	0.11	0.37	**0.04**
	♂ Infected		**0.04**	**0.003**
	♀ Uninfected			0.2

In former studies, Quinn and Hews (2003) and Cox et al. (2008) stressed the positive relation between levels of testosterone and the conspicuousness of the blue ventral coloration of fence lizards. Therefore, fainted blue ventral patches showed by male lizards infected by *Acroeimeria* parasites might reflect lower levels of testosterone implying detrimental effects of infection over the fitness of these males (Dunlap and Mathies, 1993; Dunlap and Schall, 1995). Indeed, lower basal levels of testosterone and high corticosterone levels were found in fence lizards infected by malarial parasites in comparison to uninfected ones (Dunlap and Schall, 1995). Therefore, duller blue coloration of infected males may be related with lower levels of testosterone and, consequently, result in a lower number of social interactions and less aggressive behavior in male fence lizards thus reducing their accessibility to potential mates (Moore and Marler, 1987; Schall and Dearing, 1987; Schall and Sarni, 1987; Schall and Houle, 1992). Alternatively, males with female-like levels of blue chroma may benefit by lower aggressiveness by dominant males (Cooper and Burns, 1987), allowing subordinate males sneaking into female territories more easily and hence, favoring the transmission of the parasite and explaining the maintenance of both aggressive and submissive sexual strategies in this species.

The role of *Acroeimeria* parasites biasing the chromatic dimorphism in this population was supported by the results in relation to the yellow patch on the forelimbs. The females infected by *Acroeimeria* showed brighter forelimbs than the infected males. The brightness of the forelimbs was associated with worse body condition in either sex. In this sense, the body condition of the females in *Sceloporus virgatus* was positively associated with antioxidant deposition in the clutch (Weiss et al., 2011). Thus, brighter females with worse body condition might be signaling to

conspecifics their individual quality and health status. Furthermore, bright yellow or orange ornaments in females of phrynosomatids are associated with behaviors of rejection against candidate males that try to mate with them (Clarke, 1965 in Cooper, 1987; Cooper 1984; Cooper and Crews, 1987; Hager, 2001). One plausible hypothesis is that the rejection mechanism may avoid weakened or non-receptive females the inherent costs of mating or reproduction (Cooper, 1986, 1987). However, brighter females received greater attention by courting males in phrynosomatid species (Clarke, 1965; Cooper, 1984, 1988; Calisi et al., 2008). Thus, a striking alternative hypothesis is that the aggressive behavior of females against candidate males ensures that only the more persistent and thus, best quality males get access to the females (e.g. Calisi et al., 2008; Chan et al., 2009). If more persistent males got access to the weakened but brighter females, their genes would pass onto the next generation, and thus, if females withstood the costs associated with reproduction (e.g. Sorci et al., 1996), they will benefit by pairing with such males transmitting genes of resistance to parasitic disease to their offspring.

In opposition to *Acroeimeria*, parasites of the genus *Schellackia* were found significantly more often in males than in females. In fact, only two females were infected by *Schellackia* making difficult to test the relationship between this parasite and the coloration of females. This sexual difference in the prevalence of *Schellackia* parasites suggests a higher susceptibility of the males to get infected either by the parasite or by the pterygosomatid mites that transmit the protozoa (see Klein et al., 1988). This result may be in line with the immunocompetence handicap hypothesis (ICHH, Folstad and Karter, 1992). In this sense, there is some supporting evidence of the immunomodulation effect of testosterone in reptiles (Belliure et al., 2004; Roberts et al., 2004 but see Veiga et al., 2003; Oppliger et al., 2004). Indeed, seasonal and experimental peaks of testosterone were associated with increased number of attached ectoparasites in lizards of different families (Salvador et al., 1996; Olsson et al., 2000; Uller and Olsson, 2003; Klukowski, 2004; Cox and John-Alder, 2007; Halliday et al., 2014). The increase in the number of ectoparasites attached to the skin of male lizards during the mating season may increase the chances of getting infected with mite-born protozoa. Male lizards infected by *Schellackia* parasites in our study showed significantly darker blue patches and higher chroma in the yellow patch of the forelimbs than the uninfected males. Similarly, males of *S. occidentalis* infected with malaria parasites showed darker ventral coloration than uninfected ones (Ressel and Schall, 1989). These results suggest that parasites might somehow be associated to the balance of hormone levels in *S. occidentalis bocourtii*. ,Unfortunately, we did not measure testosterone levels in these lizards so we cannot conclusively support/reject ICHH. However, the conspicuousness of melanin- and pteridine/carotenoid-based ventral patches of phrynosomatids (Cox et al., 2005; Weiss et al., 2012) may depend on circulating testosterone levels (Kimball and Erpino, 1971; Rand, 1992; Quinn and Hews, 2003; Cox et al., 2005, 2008; Calisi and Hews,

2007). In fact, experimentally castrated males have reduced conspicuity of the blue ventral patch that was recovered after supplying them with testosterone implants (Cox et al., 2008). Formerly, the testosterone-treated lizards of a previous experiment increased the density of melanin in the melanophores (Quinn and Hews, 2003). When the density of melanin was augmented in the blue ventral patch, the chroma and the hue increased, but the brightness decreased (Cox et al., 2008). In our study, males with darker blue ventral patches infected by *Schellackia* might be reflecting higher concentration of melanin in the melanophores (Cox et al., 2008). The eumelanin is the main type of melanin in the skin of reptiles (but see Roulin et al., 2013) and its synthesis and deposition is favored under oxidant conditions (Galván and Solano, 2009, 2015). Such conditions may also be promoted by parasites, which might deplete the glutathione availability (Atamna and Ginsburg, 1997; López-Arrabé et al., 2015), favoring eumelanogenesis (Galván and Alonso-Álvarez, 2008). In this sense, the pro-oxidant properties of the testosterone (Alonso-Álvarez et al., 2007) may also contribute to eumelanogenesis (Adachi et al., 2010) and hence, to the conspicuousness of sexual characters (Folstad and Karter, 1992; Mougeot et al., 2009). Indeed, melanin deposition in basal layers of the skin in combination with xanthophores and iridophores may increase the chroma of either blue and orange/yellow ornaments of ectotherms (Grether et al., 2004). For example, in *S. pyrocephalus* where females are the ornamented sex, females expressed more saturated red gular pigmentation with experimentally increased testosterone levels (Calisi and Hews, 2007). In other experiment, males of *S. undulatus* experimentally treated with testosterone increased the expression of their yellow/orange and blue ornaments after 21 days of treatment (Rand, 1992).

In conclusion, the changes in chroma, hue and brightness of ventral color patches in either sex of *S. occidentalis bocourtii* may indicate multiple parasitic infections. In addition, the chromatic differences between uninfected and *Acroeimeria*-infected individuals are sex dependent suggesting that this parasite may alter the chromatic dimorphism in *S. occidentalis bocourtii*. To understand whether the parasite-induced changes in color patches alter visual signals and influence mate choice as well as other social interactions in this species, future studies on testosterone levels and social interactions of parasitized and non-parasitized individuals will be necessary.

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INTEGRATIVE DISCUSSION

In order to study the host-parasite interactions of a system we need first to clearly identify the species implied. This is important for several reasons including to be aware of the effect of interactions between parasite species to the health, development and behaviour of the host, to identify the effect of each species on each host, to note the specificity of each parasite which may affect the probabilities of transmission and thus the effect on the host population or to note the effect of a parasite species on the other. Unfortunately, there are some groups of vertebrates that are still poorly explored in terms of the parasite species infecting them and lizards are one of these groups without any doubt. When I first approach the study of parasites in lizards to study the ecology of their interactions I discover soon that I should be able to identify species that were under-described or unknown and my first step was trying to identify correctly these species. A task of some months soon was converted in an important part of my thesis dissertation because of the state of the art in this area of parasitism. For example, a common browser used in research found 1.330.000 cites when the word "parasite" was used as searching criterium. The number of references found was 88.300 when "bird" was added to the word "parasite". If we included "parasite" and "mammal" we got 64.100. Finally, if we included "parasite" and "lizard" we got 13.900 results. Thus, so far the effort to study host-parasite interaction in these vertebrates is quantitatively lower than in other groups of vertebrate hosts. Indeed, previously to the present investigation the number of available sequences of parasites within Eimeriorina that infect lizards was two. In this sense, as a consequence of the effort to identify and classify correctly parasites of lizards 7 new species were described and their phylogenetic position and evolution clarified based on 37 newly characterized sequences of parasites within the genera *Acroeimeria* (2); *Caryospora* (1), *Choleoeimeria* (3), *Eimeria* (i.s.) (2), *Isospora* (9), *Lankesterella* (2), and *Schellackia* (18). This work allows me to confront a better study of the effect of several parasites on lizard ornaments completing the initial targets of my dissertation work. Different parasites may affect differently to several aspect of the physiology of colour in the skin of reptiles and the knowledge of these mechanisms is also essential to understand how parasites may affect these ornaments. In this sense experiments to modify the structure of the skin of lizards and previous knowledge on the effect of parasites on lizards allow me to understand how the effect of parasites modulates sexual signalization in species under study. Therefore we group discussion around the two following chapters, the first on evolutionary relationships of coccidian parasites and the second around the signaling of lizards in relation with parasitism.

Chapter I: *Evolutionary relationships of coccidia infecting lizards*

The implementation of molecular tools in the last years led to a growing assessment of the existing diversity in different taxonomic groups where cryptic species remained to be discovered (e.g. Horton and Bruns, 2001; Godfray, 2002; Anderson and Cairney, 2004; Vieites et al., 2009; Geniez et al., 2014). Indeed, characterization of new taxa using molecular techniques is particularly useful in the systematics of unicellular or simple organisms where morphological characteristics are scant (Perkins, 2000; Ghimire, 2010). In this sense, the description of new taxa of symbiotic organisms such as parasites increases the number of species in a given area. This fact increases our responsibility to protect and to preserve species that at the same time are harboring infra-communities of specific-dependent organisms (Guégan and Hugueny, 1994; Graham et al., 2009). Such is the case of the coccidian parasites that infect lizards. However, the information on this group is scarce and is common to find general designations for these organisms. Indeed, a common term to designate these parasitic organisms is hemogregarine or haemogregarine referred to parasites found in blood cells in circulating peripheral blood of reptiles. This term is not exact, since *Haemogregarina* (Apicomplexa: Haemogregarinidae) is a genus of hemoparasites found in reptiles and other ectotherms and it is especially misleading in Spanish since the spelling is "hemogregarina". As commented in the introduction of this dissertation, Siddall (1995) and Smith (1996) proposed to include all parasites of unknown life cycle found in reptiles, formerly classified in the genus *Haemogregarina*, in the genus *Hepatozoon* (Adeleorina). Additionally in 1920 *Karyolysus*, a genus of hematic parasites commonly found in the blood of European lizards, had been newly described (Reichenow, 1920a; Svahn, 1974; Haklová-Kočíková et al., 2014). These adeleorine parasites are particularly abundant in the blood of lacertids with intensities up to 3% (pers. obs.) and they are fairly common in some populations of lizards as highlighted by Amo et al. (2005a, b, c); Maia et al. (2012); and Harris et al. (2012). However, a recent study highlighted the difficulty to correctly separate the genera *Hepatozoon* and *Karyolysus* based on the current molecular markers used to infer evolutionary relationships within the Adeleorina (Haklová-Kočíková et al., 2014). Therefore, an alternative to designate these *Haemogregarina*-like parasites may be just Adeleorina or adeleorine parasites until further molecular information were available to disentangle the phylogenetic affinities of these parasites.

In addition to these adeleorine parasites, there are other genera described in lizards that belong to the suborder Eimeriorina that may be found either within peripheral blood cells or passing with the feces. The present dissertation focused on exploring, for the first time, the evolutionary relationships among the eimeriorine genera *Schellackia*, *Lankesterella*, *Caryospora*, *Isospora*, *Choleoeimeria* and *Acroeimeria* that infect lizards using *18S* rRNA gene sequences. In this sense, although some authors suggest using faster evolving genes (e.g. mitochondrial genes) to study phylogenetic affinities among the closely related Adeleorina (Barta et al., 2012; Haklová-

Kočíková et al., 2014), previous studies using nuclear *18S* rRNA gene sequences for the study of the suborder Eimeriorina demonstrated that this marker is appropriate and highly informative (Zhao et al., 2001; Zhao and Duszynski, 2001; Ogedengbe et al., 2015). Therefore, in the present investigation we used *18S* rRNA gene sequences to molecularly characterize and infer phylogenetic affinities among eimeriorine parasites. Indeed, using this genetic marker we were able to note that the original description of *Schellackia bolivari* Reichenow 1920 was based on a mixed description of the endogenous and the exogenous life stages (Figure 1) of two taxa that belonged to different genera (*Lankesterella* and *Schellackia*) (Megía-Palma et al., 2014). Additionally, we provided data highlighting the molecular diversity within the genus *Schellackia* that parasitizes lacertids from the Iberian Peninsula. All these data may contribute in the future to describing new taxa and to the enrichment of the knowledge on Iberian Peninsula biodiversity.

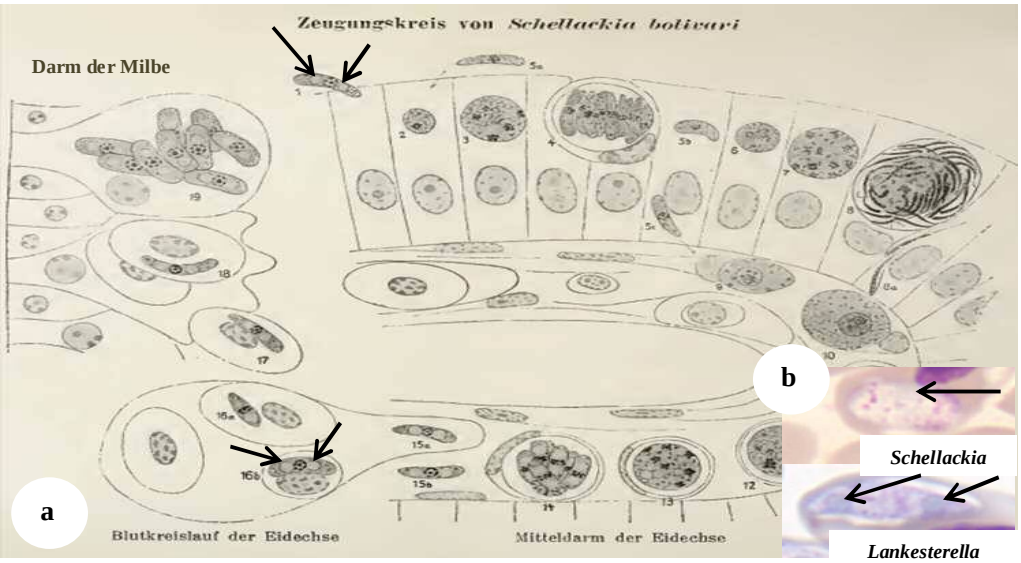

Figure 1. (**a**) *Schellackia bolivari*, type species for the genus, originally described in *Acanthodactylus erythrurus* (Lacertidae). Merozoites, gametocytes and sporozoites show two refractile bodies (black arrows). Line drawings from Reichenow 1920b. (**b**) In Megía-Palma et al., 2014 hematic stages with two refractile bodies (RB) grouped with *Lankesterella* species, whereas hematic stages with one RB grouped with parasites of the genus *Schellackia*.

In the first chapter of this dissertation (studies 1, 2 and 3), hemococcidia parasites of the genera *Lankesterella* and *Schellackia* that infect lizards were molecularly characterized for the first time. The hemococcidia (Eimeriorina) is a designation that refers to the genera *Schellackia* and *Lankesterella* which are considered uncommon or innocuous parasites in natural populations of lizards. In particular, the sporozoites of the parasites within the genus *Schellackia* that infect the cytoplasm of host blood cells are usually found in intensities of about 0.001%. Thus, is reasonable to count at least 15.000 cells prior to diagnose an individual as negative for infection by *Schellackia*. In addition, the sporozoites of the parasites within the genus *Schellackia* that are

found in the blood cells of host lizards are often difficult to identify because they are distinguishable only by particular differences with those within the Adeleorina: 1) mature gamonts of adeleorine parasites are surrounded by an often patent parasitophorus vacuole, 2) hematic stages of parasites in the genus *Schellackia* (sporozoites) do not distort the nucleus, 3) these sporozoites do not change the shape or the size of the host cell, and most important 4) mature sporozoites of *Schellackia* parasites found in blood host cells commonly show refractile bodies that are faintly stained with Giemsa and are distinguishable by optic microscopy (Telford, 2008). The refractile bodies in mature sporozoites of hemococcidian parasites may not be confused with vacuoles in immature stages of adeleorine parasites (Figure 2, black arrows). These differential characteristics may be especially useful when the observer was screening blood smears infected by more than one genus of hemoparasites.

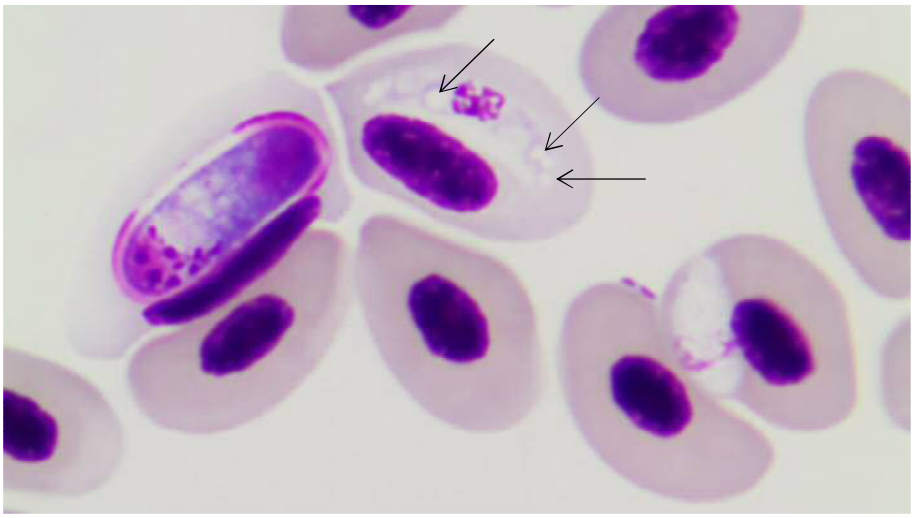

Figure 2. Mixed infection of parasites of the genera *Karyolysus* and *Schellackia* in *Podarcis muralis* peripheral blood. Blood stages of these parasites commonly infect erythrocytes in the blood of lizards. In the microphotograph, from left to right: one mature gamont of *Karyolysus* cf. *lacertae* Reichenow 1920b surrounded by a parasitophorus vacuole, one immature gamont of *Karyolysus* showing several vacuoles (black arrows). On the bottom right of the picture there is one mature sporozoite of *Schellackia* occupying an undistorted host cell.

So far, ten species within the genus *Schellackia*, and two within the genus *Lankesterella* were described from different lizard host species in the world (Telford, 2008). However, the evolutionary relationships of parasites of these genera that were found in lizards had been inferred only using consistent morphological characters as compared to other coccidia. For example, the genus *Schellackia* had been traditionally related with the genus *Eimeria* based on the presence of refractile bodies in various stages of the life cycle of parasites of both genera (Paperna and Ostrovska, 1989). In fact, the results in the studies 1 and 2 revealed the close relationship between the genus *Schellackia* and the genus *Eimeria* (Megía-Palma et al., 2013). Furthermore, based on

the presence of hematic stages in the life cycles of the genera *Schellackia* and *Lankesterella*, both had been classified within the family Lankesterellidae. However, the endogenous oocyst described for each of these genera differed in the number of naked sporozoites (Upton, 2000). In this sense, the results of the study 2 revealed that *Schellackia* and *Lankesterella* parasites had an independent evolutionary origin. In addition, the re-erection of the family *Schellackiidae* Grassé 1953 was suggested based in the monophyletic origin of the genus *Schellackia*. In the study 3, additionally, we included in the analyses *18S* rRNA gene sequences of *Schellackia* parasites isolated from 15 different species of lacertid hosts from the Iberian Peninsula and the North of Africa. In this study, the diversity of this genus was highlighted. Moreover, the specificity of these parasites was evidenced since no cross infections among host genera were detected, suggesting that the co-evolutionary relationships between these parasites and their hosts may have specific particularities.

In this clade of *Schellackia* parasites, we found two conflicting sequences. One sequence was isolated from gut tissue of European brown frogs infected with *Eimeria ranae* Dobell 1909 (Jirků et al., 2009). The second sequence came from oocysts of *E. arnyi* Upton & Oppert 1991 found infecting the North American ring-neck snake. However, the origin of the samples where the *18S* rRNA gene sequences were isolated from may be conflictive. The genetic material from *E. ranae* was isolated using gut tissue of infected tadpoles (Jirků et al., 2009). This tissue might have contained endogenous stages of *Schellackia* parasites given that these hemococcidia also infects frogs (e.g. Paperna and Lainson, 1995). In relation to *E. arnyi*, the *18S* rRNA gene sequence of this parasite was obtained from a direct submission in GenBank and remains unpublished nowhere else. Hence given the phylogenetic position of *Eimeria*-like parasites infecting lizards (Megía-Palma et al., 2015), my recommendation to achieve solid conclusions on the phylogenetic affinities of conflicting sequences like *E. arnyi* and *E. ranae* is to repeat the sampling and process of these *Eimeria*-like parasites of frogs and snakes. Other striking case of parasites with doubtful classification was the *Lankesterella* parasites found infecting polymorphic heterophils in the blood of green anoles during the surveys for apicomplexan parasites performed in this investigation. The size, the single refractile body, and the host cell type infected by this parasite in the green anoles are coincident with the formerly described *Schellackia golvani* Rogier and Landau 1975 (Figure 3) which has the Green anole among its reported hosts (Telford, 2008). Although reclassification of parasites based on molecular characterization of hematic stages of the parasite has been conducted in other cases (Merino et al., 2006; Biedrzycka et al., 2013), more evidences on the life cycle of this parasite might be needed to re-classify *S. golvani* into the genus *Lankesterella* and hence, we preferred reporting the stages found in this study as *Lankesterella* sp. *ex Anolis carolinensis*.

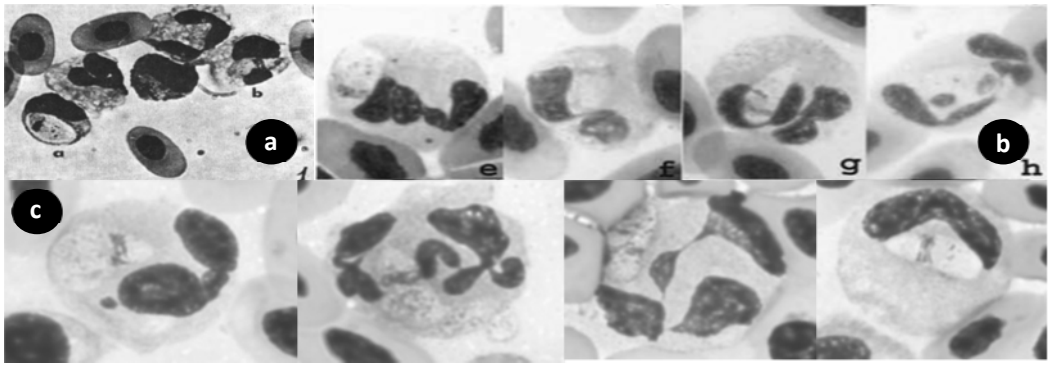

Figure 3. Microphotographs of sporozoites of *Schellackia golvani* isolated in *Anolis carolinensis* hosts in (**a**) the original description (Rogier and Landau, 1975); (**b**) in Telford's Atlas of haemoparasites of Reptilia (2008); and (**c**) *Lankesterella* sp. found in our study.

In addition to parasites of the genera *Schellackia* and *Lankesterella*, the present investigation addressed the study of the evolutionary relationships of other tissue coccidia that may undergo heteroxenous life cycles in lizards. This is the case of parasites of the genus *Caryospora* which contains four species described in lizards in the world (Upton et al., 1986; Modrý et al., 2001; McAllister et al., 2014). The inclusion for the first time of a sequence of *Caryospora* isolated in lizards, i.e. *C. ernsti* Upton et al. 1984, revealed that the genus *Caryospora* is not monophyletic. Indeed, *C. ernsti* showed a closer relation to the genus *Lankesterella* isolated from frogs, birds and lizards than to *Caryospora* parasites isolated from mice (Barta et al., 2001). Further analyses including *Caryospora* parasites isolated from birds of prey and snakes are needed to reveal phylogenetic affinities within this genus. In study 4, the inclusion for the first time of *18S* rRNA gene sequences of parasites of the genus *Isospora* found in lizards revealed the phylogenetic affinities of these parasites. Coccidian parasites with tetrazoic, disporocyst oocysts infecting vertebrates have recently been divided into different genera based on host specificity, opening sutures of the sporocyst and phylogenetic affinities (e.g. Modrý et al., 2001; Barta et al., 2005). For example, the re-erected genera *Cystoisospora* found in mammals and *Hyaloklossia* found in frogs belong to family Sarcocystidae which is the sister family of Eimeriidae and contains parasites of heteroxenous life cycles. In addition, *Atoxoplasma* was considered a genus of some parasites of birds that presented hematic stages (Barta et al., 2005; Atkinson et al., 2008). However, whether these hematic stages imply necessarily a heteroxenous life cycle remains to be clarified (see Lainson, 1960 but also Merino et al., 2006). On the other hand, the presence of hematic stages of *Isospora* parasites found in lizards similarly to *Isospora* (=*Atoxoplasma*) in birds (Barta et al., 2005; Atkinson et al., 2008) has not yet been demonstrated. However, with the information previous to the present investigation, the presence of Stieda bodies in the sporocysts of *Isospora* parasites found in both birds and lizards made likely their genetic affinity. Surprisingly, *Isospora*-like parasites found in lizards were closer related to parasites of the genera

Lankesterella and *Caryospora* than to *Isospora* parasites found in birds. Although the artificiality of the genus *Isospora* had been already demonstrated based on morphological and molecular affinities of *Isospora* (=*Cystoisospora*) isolated in mammals and *Isospora* isolated in birds (Barta et al., 2005), here we provide molecular evidence of the multiple evolutionary origins of the genus *Isospora* with Stieda bodies. Therefore, the creation of a new genus within the family Eimeriidae for *Isospora*-like parasites that infect lizards will be feasible in the future when more information on their life cycle were known (e.g. Lainson and Paperna, 1999a).

In study 5, we addressed the systematics of a particular group of eimeriids which taxonomy was controversial. Paperna and Landsberg (1989) proposed *Choleoeimeria* and *Acroeimeria* as new genera for including *Eimeria*-like coccidia that infect reptiles around the world. However, there is an open debate about the correct designation for these parasites of reptiles. The morphology of the oocyst, the presence of longitudinal sutures in the sporocysts, and the location in the body of the host where each species undergoes its endogenous development was proposed as taxonomic criteria to erect specific genera for these parasites. In fact, previous studies had evidenced a correlation between the oocyst morphology and the place in the lizard's gut where each *Eimeria*-like species undergoes its endogenous development (see Lainson and Paperna, 1999b). However, the number of intestinal coccidia of reptiles with molecular information available was only two sequences (GenBank accession numbers: AY043207 and AF324217) and no intra-clade information on the phylogenetic affinities of these *Eimeria*-like parasites that infect lizards was available. The phylogenetic analyses performed in the study 5 using *18S* rRNA gene sequences revealed the monophyletic origins of *Choleoeimeria*- and *Acroeimeria*-like parasites supporting the validity of the genera *Choleoeimeria* and *Acroeimeria* sensu Paperna and Landsberg (1989). Indeed, *Choleoeimeria*-like parasites showed oval oocysts (length/width ratio≥ 1.4), whereas parasites with *Acroeimeria*-like oocysts showed a length/width ratio of ~ 1.3.

The data provided in this chapter are quantitative and qualitative important contributions to the study of the coccidia that infect lizards. The relevancy of these results is not strictly kept within the field of taxonomy, but within an evolutionary and ecological framework. This is because classifying these parasites allows us understanding the molecular diversity, and the multiple evolutionary origins of the coccidia that infect lizards. But also allow us to consider the role of parasites in natural populations of reptiles. In this sense, being able to identify correctly the parasites in a studied population can lead us to explain better our results (e.g. type of vector implied in the association, differential effects of different parasites on hosts) or to design better experimental protocols (e.g. medication protocols, studies on different parasite interactions). Moreover, the molecular identification of exogenous stages of parasites with endogenous development avoids us euthanizing the lizard hosts, an important issue both for ethic and ecological reasons.

Chapter II: *Signaling the individual quality in lizards: Colours and parasites in different host-parasite systems*

Hamilton and Zuk (1982) proposed that parasites may influence, or even drive, the evolution of host populations through biasing the sexual eligibility towards those individuals with inheritable capability to stand or avoid parasitic diseases. Based on this prediction, the choosing sex have some cues to assess the health status of the chosen sex. In this sense, under specific environmental pressures likely parasitism, aridity, predation, food or mating resources shortage, the eligible sex may evolve exaggerated ornaments that signal the individual's quality and are favoured through sexual selection (Fisher, 1915). In this sense, colour ornaments are conspicuous traits involved in hierarchic and health signalization in vertebrates and could be used during sexual selection (e.g. Hill, 1990; Pérez i de Lanuza et al., 2014). The conspicuousness of colour ornaments of lizards is the result of the interference of the light beams absorbed and reflected from the multiple layers that compound the dermis of these vertebrates. These layers contain both reflective structures (iridophores and conjunctive tissue) and chromatophores containing pigments (carotenoids and/or pteridines, and melanins) (e.g. Olsson et al., 2013). Colour expression, i.e. disposition, consistency and reflectivity of the structures as well as the deposition and concentration of the pigments in the chromatophores, resulting in colour conspicuousness, are driven by the combination of both genetic and environmental factors (Rand, 1992; Sinervo and Lively, 1996; Alonzo and Sinervo, 2001; Bajer et al., 2012; Langkilde and Boronow, 2012; Olsson et al., 2012, 2013; San José et al., 2013; Fulgione et al., 2015; McLean et al., 2015). In this sense, the relation found between the reflectance of colour ornaments and environmental factors such as the surrounding temperature, or the oxidative status of the bearer of a specific ornament, suggests that colour patterns may reflect the individual's ability to select and maintain either optimal thermal niches or territories with good food availability (Bajer et al., 2012; Langkilde and Boronow, 2012) Additionally, they may reflect the individual's quality to face physiologically stressing challenges (Olsson et al., 2012; San José et al., 2013). Indeed, modern adaptations of the Handicap Principle (Zahavi, 1975) would relate the production of these ornaments to physiological conditions that *a priori* may be detrimental for the bearer, signaling the individual ability to cope with this handicap (Galván and Solano, 2015). As commented above, among the environmental factors that affect the expression of colour patterns in lizards, parasites were proposed as a strong selective force modeling secondary sexual ornaments in vertebrate populations (Hamilton and Zuk, 1982). In this sense, the pleiotropic adaptations on coloration to particular environmental local conditions (Ducrest et al., 2014), in the long term, may lead to phenotypical individual changes among populations subjected to different environmental pressures and thus, may lead to the loss of specific (and sexual) recognition between individuals that originally came from different populations (West-Eberhard, 1989). In turn, the loss of

specific recognition may induce a reduction in gene flow between populations driving divergence in population genetics, and eventually, speciation (Thorpe and Richard, 2001; Julienne and Glor, 2011). For this reason, studying colour expression on vertebrates in relation to different environmental conditions may be useful to understand evolutionary processes of adaptation (Reguera et al., 2014; McLean et al., 2015). Moreover, if the genetic diversity and the specificity of the coccidian parasites that infect lizards is high (chapter I), seeking for consistent patterns of relations between color expression and parasitic diseases in different host-parasite systems may help explaining common processes of adaptation to local conditions.

The second chapter of this dissertation (studies 6, 7 and 8) was focused on the relations between parasites and colour ornaments in three different host-parasite systems with specific particularities of the host mating systems. Although none of these studies was experimental, the results achieved suggest that parasites affect the expression of coloured ornaments in lizards in populations with high incidence of parasitoses. In the studies 6 and 8, we studied two lizard species that bore both blue (or UV-blue) and yellow patches. In these systems, the yellow patch was related with the body condition of the bearer and thus, this patch may be an intraspecific signal of body condition. Whereas, the blue patch in the lizard studies here was related with the presence of parasitic infections. Indeed, in lizard species where both the yellow and the blue patches were present at the same time, they may be shown synchronically during a social interaction. For example, the Schreiber's green lizard stands the head up or the Fence lizard displays standing on their limbs making visible the colourful patches. Thus, in multiple ornamented species like these ones, it is likely that multiple signals informed to potential conspecific receptors about the infection, or the susceptibility of the bearer to parasitic infections (Olsson et al., 2005a), and at the same time, it supplies information on the body condition of the bearer. In opposition, we found the striking case of *Gallotia* lizards from La Palma (study 7). In absence of a yellow patch, the blue patch gathered information on both the parasitemia and the body condition of the bearer of this signal. Thus, in populations under high incidence of parasitoses, an individual that signaled at the same time about the presence or the intensity of a parasitic infection and a good body condition might convey its capability to stand the disease (Zahavi, 1975; Hamilton and Zuk, 1982).

In phrynosomatids and lacertids, we found that patches based on different pigments reflected different parasitoses. For example, the number of ticks was negatively correlated with brightness of the yellow patch on the throat of the males *L. schreiberi*, whereas the presence of *Schellackia* parasites in the blood cells was positively related with UV-blue chroma of throats in the males from the same population (study 6). Similarly, brightness of the yellow patch in *S. occidentalis bocourtii* was related to the infection by *Acroeimeria* parasites, whereas the blue patch was related with the presence of *Schellackia* (study 8). In this sense, the metabolism of different pigments involved in visual ornamentation in vertebrates may be compromised in different ways

by different parasitoses (see McGraw and Hill, 2000; Fitze and Richner, 2002). For example, an experimental study revealed that the infection by *Isospora* parasites only affected to carotenoid-based traits in moulting birds with both carotenoid- and melanin-based ornaments (McGraw and Hill, 2000). In opposition, other experiment in a bird species with similar ornaments showed that ectoparasites of the genus *Ceratophyllus* (Siphonaptera) only affected the expression of the melanin-based trait (Fitze and Richner, 2002). Thus, a balance between parasite pathogenicity and metabolic compromises in the allocation of pigments might drive differences in phenotypic response to different parasitoses.

During the different studies of the second chapter of this dissertation, we found that the blue or UV-blue coloration was similarly related with the infection by hematic parasites. In *Gallotia* and *Lacerta* lizards the UV-blue chroma was positively related with the parasitemia and the presence of hematic coccidia respectively (studies 6 and 7). Similarly, in *Sceloporus* lizards the presence of *Schellackia* parasites was associated with darker blue ventral coloration (study 8). The physiology of the subjacent pigment involved in the blue colouration of lizards makes likely that these results were in line with the immunocompetence handicap hypothesis (Folstad and Karter, 1992). The seasonal increase in testosterone, an androgen hormone, is related with the enhancement of secondary sexual characters (Rand, 1992; Saino and Møller, 1994), but also with a negative immunomodulation and an increase in the susceptibility to parasitic infections in vertebrates (Salvador et al., 1996; Olsson et al., 2000; Mills et al., 2008; John-Alder et al., 2009; Mougeot et al., 2009). However, previous studies demonstrated that male lizards with more UV-blue reflectivity in their UV-blue visual ornaments and with better body condition have higher mating success (Martín and López, 2009; Bajer et al., 2010). Then, how do we explain that males supposedly more successful were more parasitized? UV-blue ornaments result from the combined effect of both structural and melanin deposition in the skin (Grether et al., 2004; Kuriyama et al., 2006; Olsson et al., 2013). As commented in the introduction, eumelanin is the main type of melanin known in reptiles (Ito and Wakamatsu, 2003). Melanin is stored in the melanophores of the skin of lizards which is immediately over the highly reflective underlying connective tissue. The spectral properties of the eumelanin (black pigment) makes that a high density of this pigment in the melanophores augments the purity of the wavelengths reflected by the platelets of guanine present in the layer of iridophores (Figure 5; Grether et al., 2004).

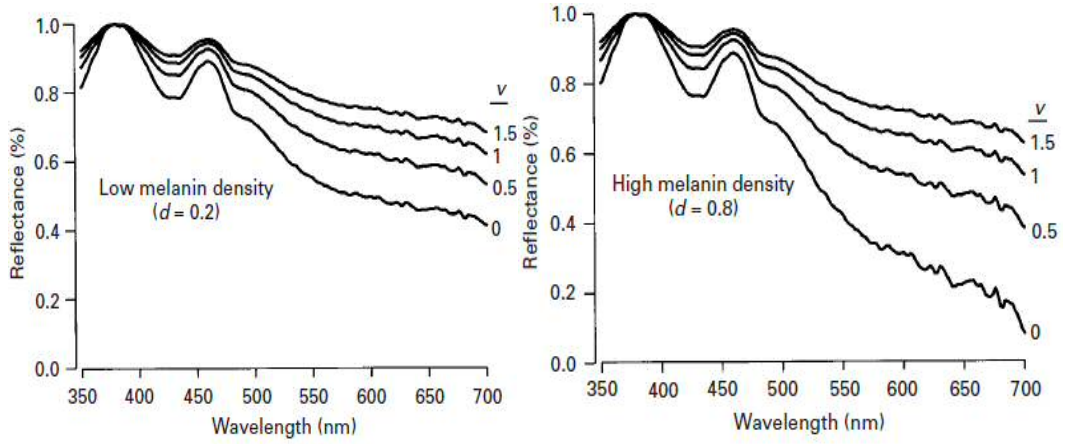

Figure 5. The effect of melanin density (d) and the amplifying effect of iridophore "blueness" (v) on the reflectance of a simulated colour patch. For this simulation, maximum iridophore reflectivity was 1; xantophore pigment was 0; and reflectivity shield present. See that with higher d the proportion of UV-blue reflectivity augments for a given value of v. Text and graph from Grether et al., 2004.

This increase in melanin concentration in the skin of lizards may reduce brightness and increase either the chroma and/or the hue of UV-blue or blue patches (Cox et al., 2008; Figure 6). In addition, the synthesis and deposition of eumelanin is favoured under both androgen (Figure 6; Quinn and Hews, 2003; Cox et al., 2005; 2008) and oxidative stress control (Galván and Alonso-Álvarez, 2008; Galván and Solano, 2009; 2015). Since reduced glutathione (GSH) is the main antioxidant molecule in eukaryotic cells (Meister, 1994), the low levels of GSH required for eumelanogenesis may handicap the bearer of the melanin-based signal (Galván and Alonso-Álvarez, 2008). However, lizards showing both strong melanin-based signals, and good body condition may be mobilizing other antioxidant molecules such as carotenoids (Blas et al., 2006; Galván and Alonso-Álvarez, 2008; Mougeot et al., 2009) conveying their individual capability to cope with oxidative stress (e.g. Roulin et al., 2011) in a Zahavi-like (1975) mechanism.

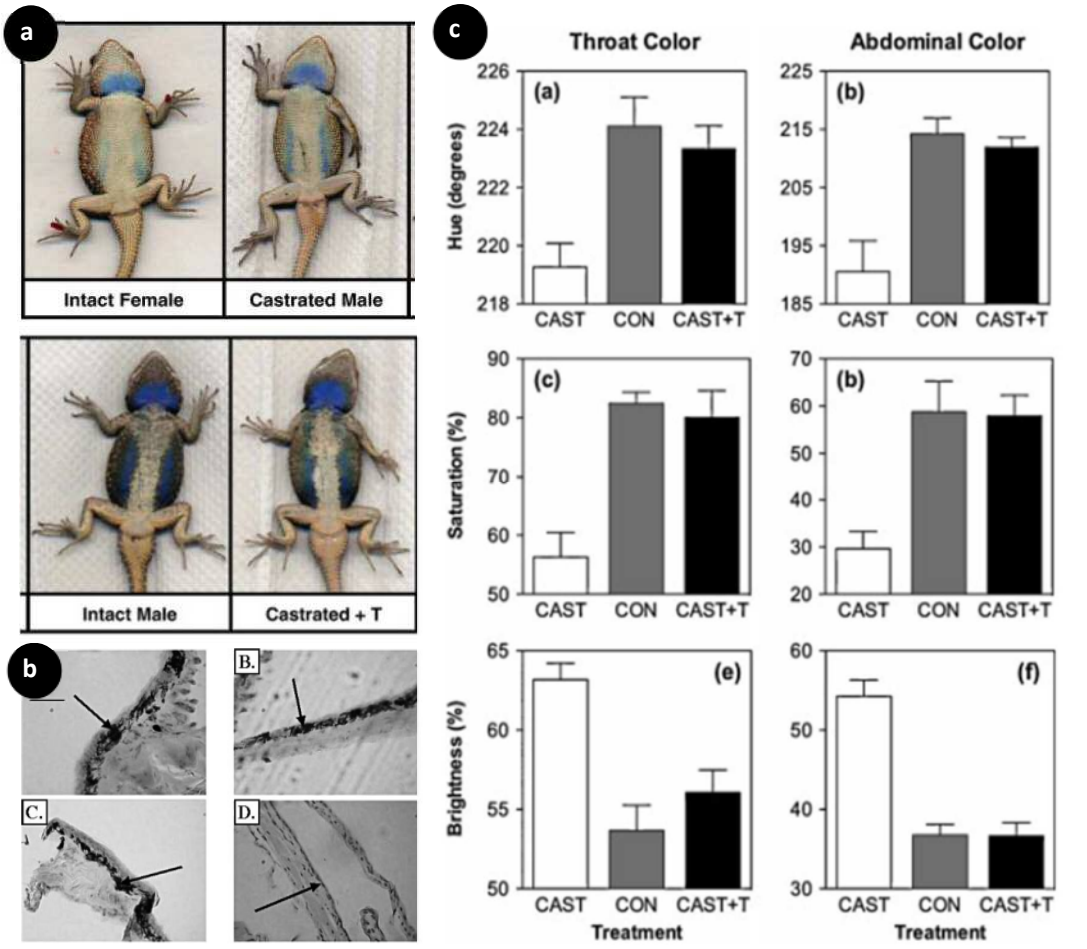

Figure 6. (**a**) Hormonal treatment with testosterone in castrated males induced re-expression of blue ornaments in *Sceloporus* male lizards. (**b**) Melanin density in melanophores in the skin of *Sceloporus* lizards. On the top left, histological cut from a male lizard, B and C experimental females treated with testosterone and 5α-dihydrotestosterone. In D: histological cut of the dermis from a control female. (**c**) An increase in testosterone induces eumelnization, in turn this increases the hue, and the chroma (=saturation) of the back and throat spectrum. However, melanization reduces brightness of lizard ornaments. Images from Quinn and Hews, 2003; Cox et al., 2008.

Additionally to the seasonal effect of testosterone, parasites may induce oxidative stress in their hosts (Atamna et al., 1997; Mougeot et al., 2009; del Cerro et al., 2010; López-Arrabé et al., 2015). Thus, the combined effect of androgen hormones and parasites may induce an increase of melanin deposition in the melanophores of the skin (Ressell and Schall, 1989). If stronger UV-blue signals in males may be associated to the presence or abundance of parasites, this supports that UV-blue ornaments in lizards are honest signals (e.g. Molnár et al., 2013). However, whether parasites directly induced high UV-blue chroma biasing the sexual eligibility of the individuals towards infected males, or alternatively, that males with higher UV-blue chroma had more social encounters with other conspecifics augmenting their chances to get infected requires further

investigation. In this sense, in an experiment male lizards were treated with testosterone and they increased their mobility, getting more attached ticks than the control group (Olsson et al., 2000). In these movements, more active males may interact more with other active males, but also increase their chances to find a sexual partner. In turn, these social encounters might augment the opportunities to get infested by mites (Figure 7).

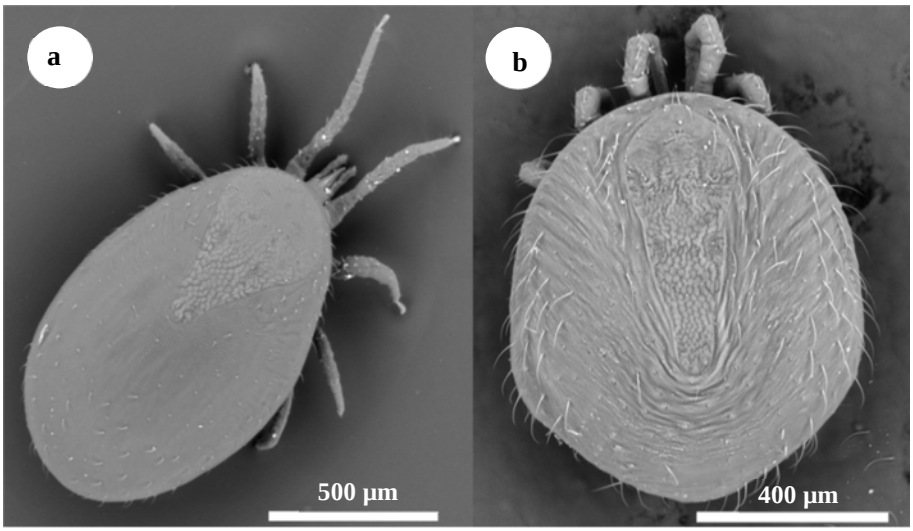

Figure 7. Mites of the genus *Ophionyssus* were described as the main transmitter of *Schellackia* and *Karyolysus* parasites in lacertid lizards. These mites may be transmitted by either contact among lizard hosts or the use of the same basking spots (Amo et al., 2005b, c) (**a**) Female *Ophionyssus* cf. *galloticolus* on *Gallotia galloti*. (**b**). Female of *Ophionyssus schreibericolus* on *Lacerta schreiberi*. SEM microphotographs by Juan Hernández-Agüero and Alberto Jorge (MNCN-CSIC).

In relation with the yellow ornaments in lizard species, males *L. schreiberi* that showed throats with brighter yellow patches had better body condition and less ectoparasites. This patch, next to the blue patch in the throat of the males of this species may act as a signal of body condition to conspecifics indicating the individual capability to allocate carotenoids from the diet into the ornamentation rather to immune functions as proposed by Hamilton and Zuk (1982). The experiment that we carried out washing the carotenoid and the melanin content out in different combinations from biopsied skin strips from lizards (study 6) indicated that negative variation in brightness of this patch may be provoked by an increase of either carotenoid or melanin concentration in the skin. The first option is unlikely, since a high oxidative challenge, like it is a high parasite load, may induce carotenoid reallocation into the antioxidant machinery rather than into ornamentation (Martínez-Padilla et al., 2007; 2010; del Cerro et al., 2010). Therefore, an acute infection provoked by ectoparasites, may induce a quick physiological response motivating the synthesis of melanin. An alternative to this hypothesis is that individuals with specific alleles of the major histocompatibility complex (MHC) that conferred resistance to the infestation by

ectoparasites were correlated with the differential expression of coloured patches. Therefore, lizards with genetic resistance to ectoparasites showed different coloured patches compared to those individuals without such alleles, as evidenced in the closely related European Sand lizard, *L. agilis* (Olsson et al., 2005a, b). The evolutionary maintenance of individuals without the specific MHC allele of resistance may be given by the handicap associated to the expression of such alleles of resistance (Olsson et al., 2005b). Thus, only good quality lizards can stand the cost associated to parasitism.

Hamilton and Zuk (1982) argued that complex displays and chromatic dimorphism might evolve in populations with high pressure of parasitic diseases. In this sense, all the systems studied in this thesis were good models to test this hypothesis since the three populations studied presented a prevalence of different parasitic diseases above 40%. A central assumption in evolutionary biology is that females of sexually dimorphic species where males are the eligible sex suffer costs when bearing male-like secondary sexual traits (Swierk and Langkilde, 2013). In this sense, we found that females of the *tizón* lizard in La Palma had worse body condition when they showed bluish cheeks similar to those in the males. However, they had better condition when this sexual ornament showed the typical whitish female-like colouration. In previous studies, masculinized females bearing testosterone-dependent traits have delayed egg-laying time (Clotfelter et al., 2004; Swierk and Langkilde, 2013), they are attacked by males or simply they are not courted, reducing their fitness (Cooper and Burns, 1987; Mokkonen et al., 2012). In addition, embryos exposed to high testosterone levels during development may be more susceptible to parasitoses than non-exposed ones (Uller and Olsson, 2003). However, there is a growing body of evidence showing that females bear ornaments with specific function (Cooper and McGuire, 1993; Irwin, 1994; Watkins, 1996; Cuadrado, 2000; Weiss, 2002, 2006; Calisi and Hews, 2007; Calisi et al., 2008; Weiss et al., 2009; Cuervo and Belliure, 2013). Thus, the correlational hypothesis that proposes that females expressed typically male traits by genetic correlation (Lande, 1980; Muma and Weatherhead, 1989) is unlikely because, as evidenced in this thesis and previous studies, producing and maintaining coloured traits is costly. On the other hand, female-specific traits may be sexually selected only if males got an advantage in terms of offspring fitness by selecting the sexiest females over other females (e.g. Weiss et al., 2009). For example, females of the Coast Range fence lizard from California (study 7) with blue ventral ornaments similar to males were infected by *Acroeimeria* parasites, which in turn was associated with weaker females that showed bright forelimbs. Indeed, infected females showed brighter forelimbs than both infected and uninfected males. In this sense, in close related phrynosomatid lizards, brighter females were more aggressive and show rejecting behaviour against candidate males of poor genetic quality (e.g. Cooper and Crews, 1987; Calisi et al., 2008). However, brighter females receive major attention in phrynosomatids (Cooper, 1988). Thus, the rejection behaviour in females may have

evolved to 1) avoid the costs of reproduction for sick, weakened or gravid lizard females (Figure 9) (e.g. Sorci et al., 1996; Watkins, 1996), or 2) to ensure that good quality genes pass to the offspring. That is, if persistent, and probably fitter males, got access to brighter females (Calisi et al., 2008; Chan et al., 2009), genes of resistance to parasitic diseases would pass onto the next generation as long as females withstood the costs associated with reproduction (Hamilton and Zuk, 1982).

Figure 9. Female *Sceloporus graciosus* showing orange colouration. This ornamentation can be observed in gravid females. Photo: Senda Reguera.

This thesis contributes with new hypotheses that may explain the relations found between colour expression in lizards and the infracommunities of parasites associated. Although is not new, the relations found here in different host-parasite system highlight that colour expression in vertebrates is influenced by multiple environmental factors. Additionally, intraspecific signals may convey the individual's ability to fit local conditions in changing environments. Further research exploring the influence of these changes on the behaviour and the sexual selection of these lizard species may be a fruitful line of investigation in the future.

CONCLUSIONS

1) The genera *Schellackia* and *Lankesterella* have independent evolutionary origins, and thus, the family Lankesterellidae has not a monophyletic origin

2) The genus *Schellackia* is more diverse and host specific than it was previously known. Indeed, different host lacertid genera from the Iberian Peninsula did not share parasite haplotypes even though some of these lacertid species are sympatric.

3) *Isospora*-like parasites isolated from reptiles are not closely related to *Isospora*-like parasites from birds or mammals. They may be a completely new genus of coccidia.

4) The genus *Caryospora* has not a monophyletic origin. This was evidenced when we characterized an isolate from lizards and it was related closer to genus *Lankesterella* than to *Caryospora* parasites found in mice.

5) Parasites found in reptiles with *Eimeria*-like oocysts form a monophyletic clade. In addition, phylogenetic analyses validate the genera *Acroeimeria* and *Choleoeimeria* previously proposed by Paperna and Landsberg (1989) based on morphologic characteristics of the oocyst stage.

6) The relations found between the blue coloration with either the presence or the load of endoparasites in different host parasites systems are compatible with a higher deposition of eumelanin in the skin of the lizards. Given that high oxidant conditions are required for the synthesis of eumelanin, UV-blue or blue signals are likely to be related with the individual ability to cope with oxidative balance similarly to other vertebrate systems that also show melanin-based traits.

7) Yellow ornaments can be affected by either chronic (endoparasites) or acute and seasonal infections (ectoparasites).

8) In host species where both sexes show similar sexual ornaments, the phenotypic response to parasitic infections can be in opposite direction.

9) In dimorphic species, individuals bearing typical characteristics of the other sex are handicapped. This is the case of “bearded ladies”, meaning females with typical male-like traits. For example, females of the American lizard, *Sceloporus occidentalis bocourtii*, and the Canarian lizard, *Gallotia galloti palmae* were in better body condition or were less often parasitized when they showed typical female-like traits. In turn, males with more conspicuous color traits typical of dominant males reflect better individual quality in line with a Zahavi’s handicap-like mechanism.

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